University of Alberta

Quantification of Antibiotics in Wastewaters by Solid Phase Microextraction and Solid Phase Extraction

by

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Abstract

Solid phase extraction (SPE) and solid phase microextraction (SPME) methods were developed for the extraction of several classes of antibiotics from wastewaters. Antibiotics from the macrolide, sulfonamide and trimethoprim classes were determined in wastewater influent and effluent taken from Gold Bar Wastewater Treatment Plant in Edmonton, Alberta using the optimized extraction techniques. Analysis was performed by liquid chromatography - tandem mass spectrometry. Of the nine target analytes, 5 were present in wastewater influent and effluent. By SPE, all 5 were quantifiable with concentrations ranging between 0.044 and 1.39 µg/L and limits of quantitation (LOQs) between 0.86 and 15 ng/L. Extraction by SPME produced measurable signals for 4 of the analytes with concentrations between 0.086 and 1.57 µg/L and LOQs between 16 and 1380 ng/L. The concentrations determined for those analytes found by both extraction techniques were in good agreement, indicating that extraction by SPME is a viable alternative to SPE.

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1 Antibiotics in the aquatic environment

1.1 Introduction

The increasing demand on the Earth's limited freshwater resources has inspired intensive investigations into the occurrence, fate and possible effects of contaminants which have been found in natural waters. One such group of contaminants, which has been the focus of research world-wide, is the pharmaceutical compounds used in human and veterinary medicine. These bioactive compounds enter the aquatic environment via sewage systems, agricultural run-off and various other routes (Daughton and Ternes 1999, Halling-Sørensen et al. 1998, Richardson and Ternes 2005). Until recently, these chemicals were largely overlooked as being potentially important environmental contaminants in favor of research focusing on other classes of chemicals displaying acute toxicity and/or persistence in the environment (Daughton and Ternes 1999).

Pharmaceuticals comprise a large and diverse group of compounds with various biochemical actions. Both metabolites and unchanged parent compounds are excreted by consumers, and are subsequently discharged to wastewater treatment plants where they are subject to a number of possible chemical and biological transformations. Despite the wastewater treatment process, many compounds are not completely removed and therefore enter the aquatic environment in a continual manner via discharge of treated and untreated sewage (Daughton and Ternes 1999, McArdell et al. 2003, Weigel et al. 2004). Pharmaceutical classes that have been detected frequently in the environment include antibiotics, non-steroidal anti-inflammatory drugs (NSAIDS), β-blockers, lipid regulators and numerous others (Kim et al. 2007, Kolpin et al. 2002, Miao et al. 2004, Weigel et al. 2004). Their continual input into the environment means that a relatively constant level of contamination is maintained (Daughton and Ternes 1999). The effects of these low, but constant levels of bioactive pharmaceuticals and their metabolites to non-target organisms are not well understood, but are of significant concern. As these drugs are intended to elicit a biochemical response in target organisms (humans, livestock, fish), it is alarming to consider the potential implications for non-target organisms subjected to constant exposure to a wide array of these chemicals. Since the level of exposure is so low, acute toxicity to non-target organisms is not expected to be as important as chronic toxicity (Fent et al. 2006). Subtle effects resulting from long-term, constant exposure to low levels of these chemicals is the foremost concern (Fent et al. 2006, Halling-Sørensen et al. 1998). Adequate methods for determining the effects of chronic exposure to low levels of these chemicals over a prolonged period of time are not well established, so it is difficult or impossible to distinguish between natural change to an ecosystem and its inhabitants, and change that is a result of exposure to these contaminants (Fent et al. 2006). Furthermore, contamination of drinking water poses a potential risk to human health, as the effects of constant ingestion of sub-therapeutic doses of pharmaceuticals over extended periods of time are not known. An additional concern for the antibiotic class of pharmaceutical compounds is the development of antibiotic resistance by microbes in the aquatic environment. Potential development of antibiotic resistance by harmful pathogens because of antibiotic presence in the environment represents a serious health threat to humans and other organisms (Kümmerer 2004). Increased incidence of microbial antibiotic resistance has been found in both wastewater (Auerbach et al. 2007, Xu et al. 2007a) and aquatic environments impacted by wastewater (Goñi-Urriza et al. 2000, Huddleston et al. 2006). Thus, environmental waters may serve as a reservoir for harmful antibiotic-resistant microbes

(Huddleston et al. 2006). The presence of antibiotics in the aquatic environment has also shown potential to impact aquatic organisms (Halling-Sørensen 2000, Isidori et al. 2005, Jones et al. 2004). Chronic toxicity of low concentrations of human-use antibiotics present in the aquatic environment has been demonstrated for some aquatic organisms; in particular, alga shows sensitivity toward many antibiotic compounds (Isidori et al. 2005, Yamashita et al. 2006).

The issue of pharmaceuticals in environmental waters is complex and multi-faceted, and knowledge is still limited with regard to occurrence, fate and effects of these compounds in the aquatic environment. Research aiming to understand the various aspects of this issue is on-going, and much more is understood about the subject compared to about a decade ago when the situation was first given serious consideration (Daughton and Ternes 1999, Halling-Sørensen et al. 1998).

To gain insight into the fate and occurrence of these compounds, is it essential that sensitive and reliable analytical techniques are established to measure them. With the increased availability of analytical instrumentation that is sufficiently sensitive to detect polar pharmaceutical compounds at the low levels (ng/L–µg/L) typically found in the environment, detection and quantification of these analytes in a variety of aqueous environmental matrices is done with increasing regularity. Analytical methodology for the extraction and quantification of a diverse array of pharmaceuticals in aqueous environmental matrices is readily available in the literature, and numerous reviews are available on the subject (Fatta et al. 2007, Hernández et al. 2007, Ternes 2001).

Several extraction techniques have been employed for the extraction of pharmaceuticals from aqueous matrices, the most popular of which is solid phase extraction (SPE) (Hernández et al. 2007). Detection by liquid chromatography - tandem mass spectrometry with electrospray ionization is becoming the standard in analytical instrumentation because of its high sensitivity, and amenability to the analysis of polar and/or thermally labile compounds (Fatta et al. 2007, Hernández et al. 2007).

The work presented in this thesis details the development of an extraction technique utilizing the newer solid phase microextraction (SPME) technology. Solid phase microextraction offers potential advantages over other extraction techniques (i.e., solid phase extraction) used for the collection of antibiotics in waste and environmental waters. Among these advantages are decreased sample preparation, simpler and faster extraction procedures, and decreased (or elimination of) matrix effects in analysis. In this work, a solid phase microextraction method was optimized for the extraction of antibiotic compounds from wastewater influent and effluent. As antibiotic compounds were chosen for investigation for this thesis, discussion will be limited to these compounds.

Chapter 1 is an introduction to the issue of antibiotics in the aquatic environment. In this section, a review of select literature on occurrence, fate and effects of antibiotic compounds in the environment is presented. A discussion of the analytical techniques used to measure these compounds in the aquatic environment is also presented.

In Chapter 2, the research undertaken to optimize and apply a solid phase microextraction technique to the extraction of wastewaters in Alberta, Canada, is detailed. A total of 9 target compounds from the macrolide, sulfonamide and trimethoprim classes of antibiotics were chosen for investigation based on previous studies showing their presence in waste and surface waters in North America (Kolpin et al. 2002, Miao et al. 2004). The compounds investigated were the sulfonamides sulfapyridine, sulfamethoxazole, sulfamethazine, sulfadimethoxine, and sulfisoxazole, the antibiotic trimethoprim, and the macrolides erythromycin, clarithromycin and roxithromycin. Optimization and application of the SPME technique and the high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method used to quantify the collected antibiotics are discussed in detail. Results from extractions performed by both SPME and SPE on wastewaters from Edmonton Gold Bar Wastewater Treatment Plant in Edmonton, Alberta, are presented and discussed. Lastly, concluding remarks and comments on future directions of the research are summarized.

1.2 Uses of antibiotics

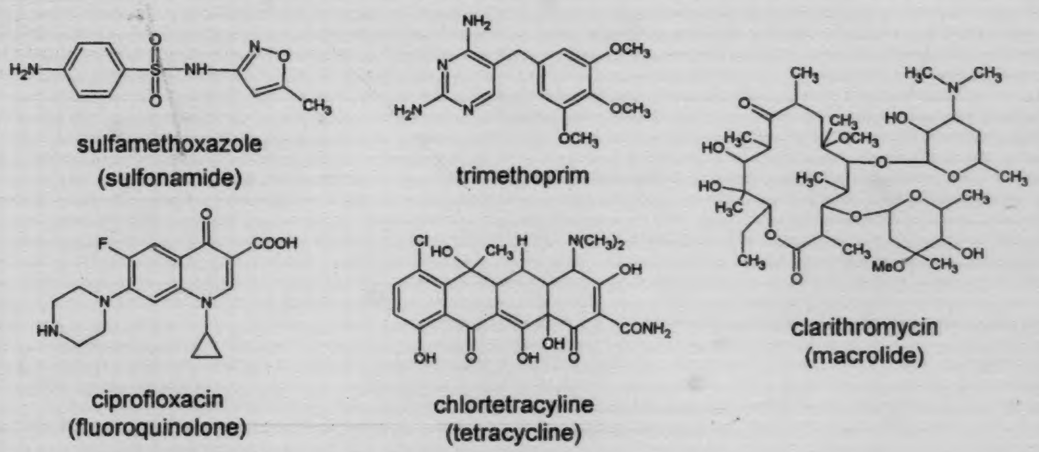
Antibiotics are molecules which are used extensively to treat bacterial infections in both humans and animals. They are also heavily used for prophylaxis and as growth promoters in food animals, and in the prevention and treatment of infection in the aquaculture industry. In 2006, antibiotics were the sixth most commonly prescribed therapeutic class in Canada (IMS Canada 2006). Major classes of antibiotics in human and animal use include macrolides, sulfonamides, trimethoprim, fluoroquinolones, tetracyclines, lincosamides and β-lactams (penicillins, cephalosporins). The latter three classes (tetracyclines, lincosamides, β-lactams) were not studied in this thesis and will not be further discussed in this section.

The macrolides are broad spectrum antibiotics comprised of a macrocyclic lactone ring attached to sugar moieties (Figure 1.1). Commonly used macrolide antibiotics include: roxithromycin, erythromycin, azithromycin and clarithromycin. They are active against many Gram-negative and Gram-positive bacteria and are used to treat conditions including respiratory, genital, gastrointestinal and skin/soft tissue infections (Jain and

Danziger 2004). They are particularly useful in the treatment of repiratory tract infections, as they tend to accumulate in respiratory tissues (Jain and Danziger 2004).

Sulfonamide antibiotics are synthetic bacteriostatics possessing a benzene ring, an amine moiety and a sulfonamide group (Sarmah et al. 2006). Figure 1.1 shows a representative sulfonamide, sulfamethoxazole. Sulfonamide antibiotics are employed in both human and veterinary medicine. In humans, sulfonamides are used frequently for the treatment of urinary tract infections, and to a lesser extent in upper respiratory infections and otitis media (infection of the middle ear) (Guardabassi and Courvalin 2006).

Figure 1.1: Representative compounds from common-use antibiotic classes



sulfamethoxazole (sulfonamide)

trimethoprim

clarithromycin (macrolide)

ciprofloxacin (fluoroquinolone)

chlortetracyline (tetracycline)

Trimethoprim is a synthetic antibiotic that is used for the treatment of urinary tract infections (Figure 1.1). It acts synergistically with the sulfonamide sulfamethoxazole, and a combination of the two drugs is commonly used to treat urinary tract infections (Walsh 2003).

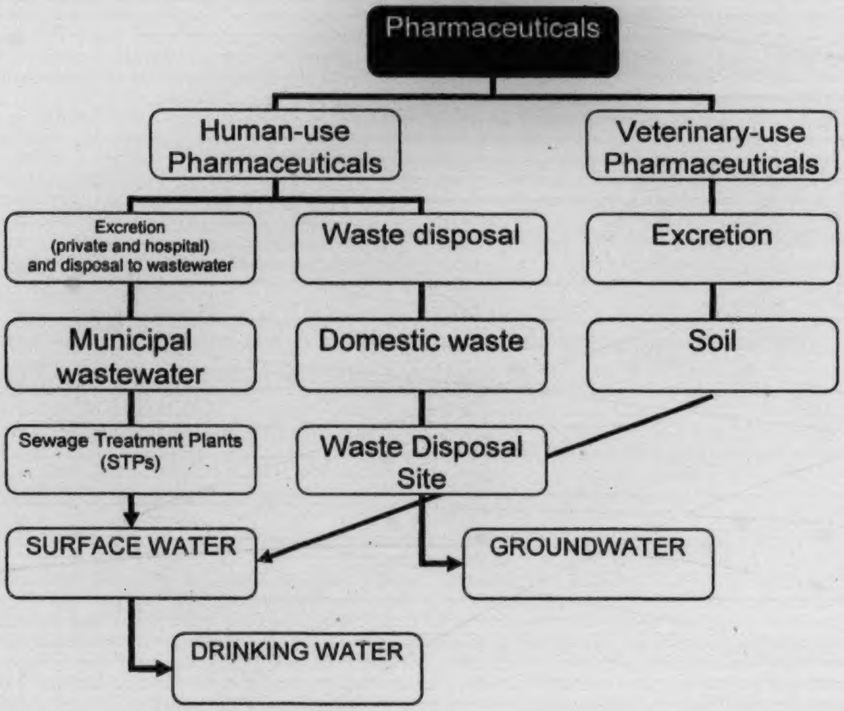
The fluoroquinolones are a class of broad spectrum antibiotics derived from nalidixic acid (Figure 1.1). They are commonly used to treat urinary tract infections, bacterial diarrhea and some sexually transmitted diseases. Newer fluoroquinolones are important in the treatment of respiratory infections (Christian 1996, Zhanel et al. 2002).

1.3 Pathways to the aquatic environment

Some of the various routes by which antibiotics and other pharmaceuticals can enter the aquatic environment are outlined in Figure 1.2, and include: wastewater treatment plant discharge to surface waters, disposal of antibiotics to landfills, and run-off from agricultural use lands into surface waters. The most significant route is via wastewater treatment plant (WWTP) discharge into surface waters (Bound and Voulvoulis 2005, Jones et al. 2005, Williams 2005), thus WWTPs can be considered important point sources for antibiotic contamination of natural waters (Ternes et al. 2006). Antibiotics are routed to WWTPs in several ways, the primary route being via human excretion of both parent compounds and metabolites. Sources to WWTPs include not only residential sewage but also hospital sewage, which has been shown to contribute to the loading of drugs to the sewage system (Brown et al. 2006, Kümmerer and Henninger 2003, Lindberg et al. 2007). In addition, the disposal of unused or expired drugs into the domestic waste stream can introduce parent compound to the wastewater treatment facilities.

There are numerous accounts in the literature of incomplete removal of antibiotics and other pharmaceuticals during the wastewater treatment process (Bendz et al. 2005, Brown et al. 2006, Göbel et al. 2007, Yang et al. 2006, Yasojima et al. 2006). The removal of antibiotics during the treatment processes in a WWTP varies greatly, with a high removal rate observed for some compounds and significantly lower removal observed for others. Thus, a significant amount of the pharmaceutical compounds and their metabolites are continuously discharged into surface waters via wastewater effluent. Removal rates are not only compound dependent, but are also dependent upon the individual treatment facility (e.g., primary versus tertiary treatment) and other factors such as rainfall and temperature (Bound and Voulvoulis 2005). Historically, wastewater treatment processes were not designed to remove pharmaceuticals specifically (Joss et al. 2006). Now that the issue of incomplete removal of pharmaceuticals during the wastewater treatment process is more widely recognized, research investigating effective removal processes is underway. The utility of, for example, advanced oxidation processes and membrane bioreactors in removing pharmaceuticals is under investigation (Dalrymple et al. 2007, Kimura et al. 2007).

Figure 1.2: Pharmaceutical pathways to the aquatic environment


Pharmaceuticals
Human-use Pharmaceuticals
Veterinary-use Pharmaceuticals
Excretion (private and hospital) and disposal to wastewater
Waste disposal
Excretion
Municipal wastewater
Domestic waste
Soil
Sewage Treatment Plants (STPs)
Waste Disposal Site
SURFACE WATER
GROUNDWATER
DRINKING WATER

Wastewater treatment facilities are often not designed to cope with heavy rain events, and excess raw sewage is discharged to receiving waters prior to any treatment (Ternes et al. 2006). In addition to contributions from large, centralized WWTPs, the facilities serving smaller communities must also be considered. In areas with low population density, private, onsite wastewater systems (e.g., septic tanks) are commonly employed in lieu of any kind of centralized WWTP, and both surface and ground waters can be contaminated by the resulting effluent. In smaller communities, the wastewater treatment facilities are generally simpler (e.g., only primary treatment) than those found in larger urban areas; consequently, the extent of pharmaceutical removal may differ (Nikolai et al. 2006).

The biosolids that are a product of wastewater treatment are commonly applied to agricultural lands as fertilizer. Certain antibiotics, including those from the fluoroquinolone and tetracycline antibiotic classes, have a high affinity for sorption to wastewater sludge and are therefore potentially present in the material applied as fertilizer (Jones-Lepp and Stevens 2007). These drugs may become mobile and enter the aquatic environment via leaching into groundwater and run-off into neighboring bodies of water.

Antibiotics are used extensively for agricultural purposes. They are used to treat infection in animals and are also given to food animals as prophylactics to prevent disease and for growth promotion (Guardabassi and Courvalin 2006). Excretion by animals introduces parent drugs and their metabolites to the environment. Leaching of the drugs through agricultural land introduces them to groundwater, while rain conditions can introduce the drugs to neighboring surface waters via runoff (Sarmah et al. 2006).

Other routes of entry into the environment include the direct application of antibiotics to water in the case of the aquaculture industry (Kemper 2007). The leachate from landfills has also been shown to be a potential route of contamination of groundwater by drugs (Barnes et al. 2004, Bound and Voulvoulis 2005).

All of the outlined routes to the environment lead to antibiotic contamination of surface water, ground water and, in some cases, drinking water. Their occurrence in these environments will be discussed at greater length in following sections.

1.4 Fate and transport of antibiotics in water

After consumption of antibiotics, the excreted parent compounds and metabolites are routed to the WWTP where they are, in many cases, only partially eliminated by the treatment process. It is well known that many antibiotic compounds and their metabolites survive the wastewater treatment process and are thus discharged into receiving waters. Therefore, a thorough understanding of the environmental fate and transport processes that govern the movement and transformation of these chemicals in WWTPs and in environmental waters is crucial.

The unique structures and properties of antibiotics and other pharmaceuticals are of great importance when considering the fate and transport of these chemicals in the aquatic environment. The relative complexity of pharmaceutical compounds has limited the usefulness of standardized environmental fate and transport models and testing for these compounds (Kümmerer et al. 2005). The compounds for which these models and tests were initially developed include lipophilic pesticides (polychlorinated biphenyls, PCBs; and dichloro-diphenyl-trichloroethane, DDT), petroleum hydrocarbons, and other organic contaminants which display similar fate and transport processes. The hydrophilicity and ionic nature of many pharmaceuticals distinguishes them from the hydrophobic contaminants for which much of the existing work on current environmental fate modeling is relevant. Modelling work on pharmaceutical fate in the environment is underway in various research groups (Khan and Ongerth 2004, Zukowska et al. 2006), but there are still knowledge gaps which need to be addressed (Kümmerer et al. 2005).

The environmental fate and transport of antibiotics and other pharmaceutical compounds in both wastewater treatment plants and in natural waters are dependent upon a number of key physical and chemical properties including: water solubility (S), acid dissociation constant (K_a), octanol-water partition coefficient (K_{ow}), vapor pressure (V_p) and distribution (i.e., distribution between water and solids) coefficient (K_d) (Kümmerer et al. 2005). Some of the major environmental fate processes that have been identified for antibiotics are: biodegradation, photolysis, and sorption (Kümmerer et al. 2005, Jones et al. 2005). For antibiotics, hydrolysis is generally only important for the β-lactam class, which readily undergo hydrolysis in WWTPs (Kümmerer et al. 2005). Deconjugation of sulfate and gluconoride metabolites during the wastewater treatment process to yield the parent compounds has also been observed for some pharmaceutical compounds (Jones et al. 2005). In the following sections, the most significant environmental fate processes affecting antibiotic compounds will be described in more detail.

1.4.1 Biodegradation

Biodegradation is regarded as an important process for the transformation of many organic pollutants in both wastewater treatment and in the aquatic environment (Kümmerer et al. 2005). The results of microbial biodegradation range from transformation of the original compound to corresponding metabolites, to full mineralization of a compound. The metabolites resulting from such transformations may display different properties than the parent compound, including increased stability and dissimilar behavior in the environment (Kümmerer et al. 2005). With regard to pharmaceuticals, biodegradation during the wastewater treatment process can lead to deconjugation of drug metabolites, yielding the original compound.

Alexy et al. (2004) used the Closed Bottle test to determine the ready biodegradability of 18 antibiotics representing the most important classes for human and veterinary use. The Closed Bottle test is commonly used to assess the "ready biodegradability" of a substance. In such tests, stringent test conditions are applied, and the tests are carried out with very low levels of bacterial inoculum (e.g., activated sludge). Compounds that meet the criteria for ready biodegradability are expected to rapidly degrade in WWTPs and in the aquatic environment. For the Closed Bottle test, the criteria that must be met by a compound to be considered readily biodegradable are as follows: the biodegradability of the compound, expressed as a percentage of oxygen consumed in the test vessel (theoretical oxygen demand, ThOD), must exceed 60% within a period of ten days after the oxygen consumption reached 10% ThOD. Investigated compounds which are frequently detected in wastewater included sulfamethoxazole, trimethoprim, clarithromycin and erythromycin. Experiments were done at high (mg/L) and low (μg/L) concentration levels. None of the compounds met the criteria to be considered readily biodegradable in either case. Only benzylpenicillin was biodegraded to any extent (27%) over the course of the 28 day experiment. This is in agreement with the results found by the recent study by Gartiser et al. (2007) which is described subsequently.

Gartiser et al. (2007) studied the biodegradability of 17 antibiotic compounds over 28 days using a combined Zahn-Wellens and CO_2 evolution test. A previous study by Alexy et al. (2004) showed that none of the compounds chosen for investigation in the study by Gartiser et al. met the criteria for ready biodegradability (Closed Bottle test). Usually when a compound fails to meet criteria for ready biodegradability, it is tested in an inherent biodegradability test. These tests are carried out with conditions that are more conducive to the biodegradation of a substance (e.g., higher concentrations of microbial inoculum), and substances which reach 70% or more biodegradation over the 28 day test are considered inherently biodegradable. Meeting the criteria required to pass an inherent biodegradability test indicates that a substance is non-persistent, though its degradation in the environment may be slow. The Zahn-Wellens test is the most commonly used inherent biodegradability test, and it has been suggested that a compound which displays 20–70% biodegradation during the test can be regarded as "partially biodegradable/ eliminable with indication of stable metabolites" (Beek et al. 2001). The CO_2 evolution test is another ready biodegradability test. The evolution of CO_2 in this test can be unambiguously assigned to mineralization of the test compound, and is therefore a good indicator of ultimate biodegradability of a substance. The authors' goal in this work was to exploit the advantages of both the inherent and ready biodegradability tests in order to gain deeper insight into the biodegradation of the target compounds.

To model the processes occurring during the wastewater treatment process, activated sludge from a wastewater treatment plant was used as inoculum, and the biodegradation of the antibiotics was studied over 28 days. Concentrations of 100 mg/L per test compound were used. Of the compounds studied, only benzylpenicillin sodium salt was found to be ultimately biodegraded (mineralized) with biodegradation extents of 78–87%. Amoxicillin, imipenem and nystatin were found to be partially biodegradable with the formation of stable metabolites. The authors noted that the antibiotics clindamycin, erythromycin, sulfomethoxazole and trimethoprim, which are frequently detected in wastewater effluent, showed no biodegradation in the study (Gartiser et al. 2007).

Similar studies by Al-Ahmad et al. (1999) and Kummerer et al. (2000) showed that the antibiotics cefotiam, ciprofloxacin, meropenem, sulfamethoxazole, penicillin G, ofloxacin and metronidazole also did not meet the criteria for ready biodegradability in the Closed Bottle test. Concentrations used in the experiment by Al-Ahmad et al. (1999) (cefotiam, ciprofloxacin, meropenem, sulfamethoxazole, penicillin G) ranged between 2.5–4.8 μg/L for the test compounds, and the duration of the experiment was extended to 40 days from the usual 28 days. At 28 days, only penicillin G had degraded (27%) and at 40 days, the degradation of penicillin G had increased to 35%, but no degradation of the other compounds was evident. Concentrations used in the Closed Bottle test done by Kummerer et al. (2000) (ciprofloxacin, ofloxacin, metronidazole) were between 2.8–5.4 mg/L for the test compounds. Similar to the previous study, biodegradability was assessed after the standard 28 days required for the Closed Bottle test, and again after 40 days. None of the investigated compounds met the criteria for ready biodegradability.

An investigation of the fate of tetracycline during the wastewater treatment process was carried out by Kim et al. (2005) using lab-scale sequencing batch reactors to simulate the activated sludge process. The concentration of tetracycline in the batch reactors was 200 µg/L. The biodegradation of tetracycline was not apparent after 20 days; sorption was found to be the primary removal mechanism.

The fate of three sulfonamide antibiotics (sulfamethazine, sulfamethoxazole, sulfathiazole) and trimethoprim was investigated in aerated batch reactors simulating different wastewater treatment processes (Peréz et al. 2005). Biodegradation was studied over a 54 day period for the following processes: primary treatment, activated sludge treatment (secondary treatment), aerobic nitrification (secondary treatment), and disinfection. The sulfonamide antibiotics were generally biodegradable in primary and secondary treatment processes, with the fastest rate of biodegradation observed in the activated sludge treatment. By 10 days, 7% or less of the sulfonamides remained intact after activated sludge treatment. The similar biodegradation profiles for the three antibiotics indicated that the enzymes responsible for degradation of the compounds were class specific instead of compound specific. Trimethoprim was resistant to degradation in primary treatment and activated sludge treatment, but was degraded completely within 3 days during the nitrification process. Such behavior was also observed elsewhere (Batt et al. 2006b). The authors note that aerobic nitrification is a relatively uncommon wastewater treatment process, and that the absence of this treatment will likely allow trimethoprim to pass through wastewater treatment with little, if any, elimination by biodegradation (Peréz et al. 2005). In treated effluent, only sulfathiazole and sulfamethazine were biodegradable, but biodegradation only became apparent at day 31 and 38 for respective compounds, indicating that biodegradation in final effluent is much slower, or non-existent for the investigated compounds.

1.4.2 Photodegradation

The potential for the degradation or transformation of antibiotics in surface waters by photolysis has been investigated in a limited number of studies (Andreozzi et al. 2003, Belden et al. 2007, Boreen et al. 2004, Werner et al. 2006). Environmental photochemistry studies exist mainly for the sulfonamide, fluoroquinolone and tetracycline classes, but generally information about photodegradation of antibiotics is sparse or, in the case of classes such as macrolides, absent (Boreen et al. 2003). Certain antibiotic compounds (tetracyclines, fluoroquinolones) have been studied extensively in phototoxicity studies from which the results may, to some extent, be extrapolated to environmentally relevant conditions (Albini and Monti 2003, Boreen et al. 2003).

Direct or indirect photolysis over the wavelengths of environmental relevance (290–600 nm) can lead to transformation of organic compounds, and is an important environmental fate process for such compounds (Schwarzenbach et al. 2003). Direct photolysis is the absorption of light by a molecule, leading to a transformation of that molecule. Indirect photolysis occurs by energy transfer from an excited species (e.g., constituents of natural organic matter) resulting in a transformation of the compound receiving the energy transfer (Schwarzenbach et al. 2003). Direct photolysis can also occur via a reaction with short-lived, reactive species such as hydroxyl radicals, singlet oxygen and ozone, which are generated in the presence of light (Schwarzenbach et al. 2003).

Both tetracycline and fluoroquinolone antibiotics have demonstrated high photoreactivity in environmental and other studies indicating that photolysis may be an important fate process for these compounds in the aquatic environment (Albini and Monti

2003, Belden et al. 2007, Morrison et al. 1991). The presence of chromophores presenting conjugated pi-electron systems, (e.g., aromatic rings) may indicate the susceptibility of a compound to photoreactions (Boreen et al. 2003, Schwarzenbach et al. 2003). It is therefore not unreasonable to expect that antibiotics containing such functional groups are able to undergo photolytic reactions.

Photolysis is not expected to be significant in WWTPs, because of the relatively low level of sunlight present (Jones et al. 2005). In contrast, photolysis of antibiotics in environmental waters may be significant for certain compounds. Photolysis of these compounds in surface waters may even be a more important fate process than biodegradation, as the presence of these compounds in the environment indicates their ability to evade the rigorous biodegradation opportunities present in wastewater treatment (Boreen et al. 2003).

The rate of photolysis in surface waters is dependent on numerous factors including intensity of sunlight, depth of chemical in the water, and the presence of other matter (Jones et al. 2005, Kümmerer et al. 2005). Both fluoroquinolone and tetracycline antibiotics display a strong tendency to sorb to organic matter present in natural waters, and this can limit the extent to which they are transformed by photochemical processes (Boreen et al. 2003). The remainder of this section will detail some of the environmental photochemical studies that have been performed on antibiotics.

Boreen et al. (2004) investigated the photochemical fate of five sulfonamide antibiotics (sulfamethoxazole, sulfisoxazole, sulfamethizole, sulfathiazole, and sulfamoxole) in the aquatic environment. The photodegradation observed in natural water samples was attributed entirely to direct photolysis; no evidence of indirect photolysis was present. Rates of photolysis were compound specific and dependent on season, latitude and pH of the water. Based on their studies, the following half-lives were calculated for mid-spring conditions at noon at 45° latitude, assuming no light-attenuation: sulfamethoxazole, 48 h; sulfisoxazole, 18 h; sulfamethizole, 30 h; sulfathiazole, 2.8 h. Assuming half-light attenuation (i.e., half the sunlight is attenuated), the half-lives were estimated to double. The authors concluded that sulfathiazole should be readily degraded in the aquatic environment, while sulfamethoxazole is degraded at a much slower rate (Boreen et al. 2004).

The fluoroquinolone class of antibiotics is a very photoreactive class of compounds (Albini and Monti 2003). In a recent study, Belden et al. (2007) investigated the photodegradation of the fluoroquinolone ciprofloxacin and found rapid photodegradation of the compound (half-life = 2.9 h) in water in the absence of organic material. With the addition of organic material, sorption of the compound to the particulate occurred, resulting in less overall photodegradation of the compound.

Werner et al. (2006) studied tetracycline photolysis as a function of pH, and calcium and magnesium concentration (water hardness). The rate of photolysis of tetracycline was highly dependent upon the investigated parameters. Half-life calculations based on the developed model yielded a tetracycline half-life of 44 minutes under one set of conditions (pH 8.1), and a half-life of 120 minutes with all conditions held constant but with a pH decrease to 4.4. The variation of pH affects the speciation of tetracycline, a compound with a number of ionizable functional groups. With each successive removal of a proton, the absorbance maxima of environmental importance (approximately 300 nm for tetracycline), shifts to longer wavelengths, and the rate of photolysis is increased. Increasing solution pH will therefore results in greater absorbance and higher rates of photolysis. The rate of photolysis is also increased by the presence of Ca^{2+} and Mg^{2+}; the association of tetracycline with these cations increases its absorbance at its environmentally relevant wavelength.

Andreozzi et al. (2003) investigated the photodegradation of six pharmaceutical compounds including ofloxacin (fluoroquinolone) and sulfamethoxazole (sulfonamide). Based on experiments, predicted half-lives for the two compounds were 2.4 and 10.6 days for sulfamethoxazole and ofloxacin respectively, in winter at high latitudes (50°N). Under the same conditions, carbamazapine and clofibric acid had predicted half-lives of over 100 days. The presence of nitrate ions in solution decreased half-lives for the antibiotic compounds studied. The authors speculate that the production of OH radicals resulted from photosensitzation of the nitrate ions. The OH radicals, as described previously, can react with a substrate (indirect photolysis) to transform it.

1.4.3 Sorption

Sorption processes include adsorption, where a molecule attaches to a two-dimensional surface, and absorption, where a molecule enters a three-dimensional matrix. Both are important environmental fate processes (Schwarzenbach et al. 2003). The fate and movement of organic compounds in the environment is clearly affected by the ability of a compound to sorb to solids present in that environment, and an understanding of interactions between a given organic compound and suspended particles is necessary to predict this behavior under various conditions (Kümmerer et al. 2005). In the case of uncharged, hydrophobic compounds such as DDT and PCBs, hydrophobic interactions with particles are predominant. Partitioning is described using a linear partition coefficient, K_d, which is calculated as follows:

$$K_{d,solid} = \frac{c_s}{c_{aq}} \qquad (1)$$

where c_s is the concentration of the compound on the sorbent and c_{aq} is the concentration in the water (Tolls 2001). A relationship between K_d and the organic carbon content (f_{oc}) of the sorbent exists for many of these chemicals, and a normalization of the K_d value to the organic carbon content reduces variability in data. This normalization results in the organic carbon-normalized sorption coefficient, K_{oc}.

$$K_{oc} = \frac{K_d}{f_{oc}} \qquad (2)$$

Finally, for nonionic and hydrophobic chemicals, a strong relationship between K_{oc} and the hydrophobicity of a compound, easily measured by the octanol-water partition coefficient (K_{ow}), is observed. Estimations of K_{oc} are readily obtained from measurements of K_{ow}, and the tendency of many neutral, hydrophobic compounds to sorb to organic matter in the environment is frequently estimated using the K_{ow} parameter (Tolls 2001). A general guide to sorption potential for hydrophobic compounds is as follows: $\log K_{ow} < 2.5$ corresponds to low sorption potential, $\log K_{ow} > 2.5$ and < 4.0 corresponds to moderate sorption potential, and $\log K_{ow} > 4$ corresponds to high sorption potential (Jones-Lepp and Stevens 2007).

Despite the usefulness of the K_{ow} parameter for predicting sorption potential of some organic compounds, its application to pharmaceuticals is often inappropriate (Ternes et al. 2004, Tolls 2001, Kümmerer et al. 2005). The mechanisms by which pharmaceuticals, of which many are ionizable, interact with organic matter are varied (e.g., ion exchange, surface complexation, hydrogen bonding) and therefore can not be accounted for using a predictive measurement based solely on hydrophobic interactions. The low log K_{ow} values calculated for most pharmaceuticals indicate that interactions by hydrophobic mechanisms are not likely the primary interactions. Table 1.1 shows the log K_{ow} values for selected antibiotics.

Linear free energy relationship (LFER)-based modeling work has been done by a number of research groups in order to allow prediction of pharmaceutical fate in the environment (Breivik and Wania 2003, Zukowska et al. 2006). However, further research in this area is still necessary in order to fully understand and address this need.

Adsorption to particles present in WWTPs and in the aquatic environment may lead to removal of some antibiotics and other pharmaceuticals. The extent of adsorption is dependent on a number of environmental factors including pH, which affects the speciation of a compound with acidic and/or basic functionalities, characteristics and amount of organic matter present in the environment, concentration of divalent cations (e.g. Mg^{2+}, Ca^{2+}) and ionic strength (Kümmerer et al. 2005). Sorption can be to organic material, including sewage, or to mineral surfaces, as has been elucidated for tetracyclines (Kulshrestha et al. 2004).

Table 1.1: Log K_{ow} and sorption coefficients (K_d) for selected antibiotics

(g_{SS} = grams of sludge solid; na = data not available; a = Göbel et al. 2005b; b = Joss et al. 2006)

Antibiotic	[b]log K_{ow}	K_d sludge primary/secondary (L/g_{SS})
Sulfadimethoxine	1.6	na
Sulfamethoxazole	0.89	na/0.26±0.17[a]
Sulfapyridine	0.4	na/0.30±0.15[a]
Sulfisoxazole	-1.4	na
Clarithromycin	3.16	na/0.26±0.01[a]
Erythromycin	3.06	na/0.165[b]
Roxithromycin	2.5	na
Ciprofloxacin	-1.1–0.4	2.6±1.6/26±7.3[b]
Norfloxacin	-1.03	2.5±1.5/37±13[b]
Trimethoprim	0.91	na/0.21±0.05[a]

There are limited studies which document the sorption of antibiotics to sludge during the wastewater treatment process, and for a few compounds, such as some fluoroquinolone and tetracycline antibiotics, this may lead to significant removal from the aqueous phase (Jones-Lepp and Stevens 2007). Solid-liquid partitioning coefficients (K_d) calculated for antibiotics in contact with sewage sludge reveal their tendency to sorb to sludge (Table 1.1). Sorption to sewage sludge for compounds with a $K_d \leq 0.5$ L/g_{SS} is not expected to be important (Ternes et al. 2004). From compounds listed in Table 1.1, where data is available, it is evident that only the fluoroquinolones, norfloxacin and ciprofloxacin, would be expected to sorb to sludge to any significant extent. Indeed, the tendency of these compounds to sorb to sewage sludge has been noted (Golet et al. 2002).

Kim et al. (2005) found that sewage sludge serves as an important sink for the antibiotic tetracycline. A significant portion of tetracycline was sorbed to sewage sludge during the activated sludge process, which was simulated in lab-scale sequencing batch reactors. Three operating conditions were applied to determine the impact of hydraulic retention time (HRT) and solid retention time (SRT) on tetracycline removal. The operating conditions were as follows: phase 1, HRT = 24 h; SRT = 10 days; phase 2, HRT = 7.4 h; SRT = 10 days; phase 3, HRT = 7.4 h; SRT = 3 days. Removal efficiencies were 86.4±8.7%, 85.1±5.4% and 78.4±7.1% for phase 1, 2 and 3 conditions, respectively. The effect of decreasing removal efficiency with decreased SRT (phase 3 conditions) indicates the importance of SRT for the removal of tetracycline, and possibly other antibiotics. The authors indicate that membrane bioreactor technology may become of increasing importance since long SRT and high biomass conditions can be used in this technology, and these factors may strongly affect removal of certain compounds.

Fluoroquinolones are another class of antibiotic which have been reported to sorb to sewage sludge (Golet et al. 2003). The behavior of ciprofloxacin and norfloxacin during the wastewater treatment process was studied by mass flow analysis. Fluoroquinolone mass flow was reduced by 88–92% by the overall treatment process, primarily due to sorption to sludge. Biodegradation was considered to be insignificant, thus implicating sorption to sludge as a major removal process for these fluoroquinolone antibiotics.

Göbel et al. (2005b) detected several compounds from the macrolide, sulfonamide and trimethoprim classes of antibiotics in activated sludge. In activated sludge, concentrations of the identified targets (sulfapyridine, sulfamethoxazole, trimethoprim, azithromycin, clarithromycin) ranged between average concentrations of 28 and 68 μg/kg of dry weight. Sorption constants, K_d, were calculated for those compounds found in the activated sludge. K_d values, based on the samples taken from weekly sampling trips, ranged from 208–376 L/kg (Table 1.1). According to Ternes et al. (2004), K_d values of less than 500 L/kg indicate that removal of a compound by sorption to sewage sludge will be insignificant, in agreement with the conclusion of the study authors (Göbel et al. 2005b).

1.5 Ecotoxicity of antibiotics and antibiotic resistance

A modest amount of information is available with regard to the ecotoxicology of antibiotics and other pharmaceuticals to aquatic and terrestrial organisms (Fent et al. 2006). Aquatic organisms are expected to be particularly vulnerable to the potential effects of antibiotic exposure because of their continual exposure to these drugs via wastewater discharge into the aquatic environment. Ecological risk assessment is complicated by the fact that, although some data do exist on the acute ecotoxicity of certain drugs, very little is known about their chronic toxicity to aquatic targets (Fent et al. 2006). Regulations regarding assessment of ecotoxicity of pharmaceuticals have been introduced; both European and U.S. regulatory agencies have instituted regulations which recognize the potential risk human and veterinary pharmaceuticals may pose to the environment (Fent et al. 2006). However, much of the knowledge and information required to assess the environmental impact of pharmaceuticals properly, and thus to establish regulations and guidelines for pharmaceutical levels in the environment, is not yet available (Fent et al. 2006).

Antibiotics and other pharmaceuticals are designed to affect the targeted organism in a specific manner. The mechanism of action and pathways in target organisms may not be well understood, making it even more difficult to predict how a particular compound might affect a non-target organism. The metabolites resulting from the vast array of pharmaceuticals in use further complicate the issue as the properties and biological effects of these compounds are even less understood (Ternes et al. 2006). Most of the work done to date has focused on acute toxicity using standardized tests. Little work has considered chronic exposure to the levels of drugs that would be found in an impacted aquatic ecosystem, and this is a vitally important issue that needs to be addressed (Fent et al. 2006).

The impact antibiotics may have on microorganisms in water and soil is of concern, as is the potential for antimicrobial resistance to spread because of their presence in the environment. In the remainder of this section, selected studies investigating antibiotic toxicity to aquatic organisms will be discussed. A recent study investigating antibiotic resistance in wastewater is also presented in order to illustrate the potential threat that this issue presents (Xu et al. 2007a).

Isidori et al. (2005) studied the ecotoxicity of 6 antibiotics (erythromycin, oxytetracycline, sulfamethoxazole, ofloxacin, lincomycin, clarithromycin) to non-target organisms. Acute toxicities, presented as median effective concentrations, EC_{50}, for the antibiotics were between 10.23–64.50 mg/L for rotifers and crustaceans. The EC_{50} is the concentration of a substance in an environmental medium expected to produce an effect in 50% of test organisms, under a defined set of conditions. Bacteria and fish were less sensitive. This suggests that the acute ecotoxicities of these compounds are limited, given the much lower levels of pharmaceuticals found in the environment. Chronic toxicity tests, however, revealed toxic effects to some of the target organisms at relatively low concentrations; EC_{50} values ranged between 0.002–1.44 mg/L for the compounds investigated. Alga was particularly sensitive, in agreement with previous studies (Halling-Sørensen 2000, Yamashita et al. 2006). The macrolides presented the greatest risk to aquatic organisms, based on the chronic toxicity test used in this study (Isidori et al. 2005). The EC_{50} values calculated for clarithromycin and erythromycin for the most sensitive species, the green algae *Pseudokirchneriella subcapita*, were 0.002 mg/L and 0.020 mg/L, respectively.

The fluoroquinolone ciprofloxacin impacted stream microbial communities at a concentration of 100 µg/L, in a study by Maul et al. (2006). The effects, however, occurred at four orders of magnitude above the concentrations typically found in the aquatic environment (low ng/L concentrations). The authors suggest that the impact of ciprofloxacin alone on related microbial communities is low, but that further investigation may reveal otherwise.

Another study investigating the toxicity of fluoroquinolone antibiotics (ciprofloxacin, lomefloxacin, ofloxacin, levofloxacin, clinafloxacin, enrofloxacin, flumequine) to aquatic organisms revealed their potential to impact certain organisms adversely (Robinson et al. 2005). The organisms studied are listed in order of most to least sensitive to the investigated compounds, as determined by toxicity tests, and median EC_{50} values are displayed for each species: *Microcystis aeruginosa* (cyanobacterium; median EC_{50} = 49 µg/L), *Lemna minor* (duckweed; median EC_{50} = 106 µg/L), *Pseudokirchneriella subcapita* (green algae; median EC_{50} = 7400 µg/L), *Daphnia magna* (crustacean; limited toxicity) and *Pimephates promelas* (fathead minnow; limited toxicity). Based on expected environmental concentrations of the fluoroquinolone antibiotics, only the cyanobacterium *Microcystis aeruginosa* was determined to be potentially impacted by fluoroquinolone presence in environmental waters (Robinson et al. 2005).

The antibiotic resistance of *Enterococcus* bacterial strains isolated from raw sewage and raw sewage put through a sand column, was investigated by Xu et al. (2007a). Fifty bacterial strains with resistance to various antibiotics (kanamycin, triple sulfa, tetracycline, gentimicin, novobiocin, neomycin, chloramphenicol, ampicillin, penicillin, vancomycin, erythromycin, sulfamethoxazole/trimethoprim, ciprofloxacin, ofloxacin, norfloxacin) were isolated, all of which were resistant against triple sulfa (sulfathiazole, sulfabenzamide and sulfacetamide) and sulfamethoxazole/trimethoprim. Most isolates were resistant against three to six antibiotics. None were resistant against vancomycin. One *Enterococcus* strain isolated from both raw sewage and filtered sewage had antibiotic resistances against 10 different antibiotics. The multiple antibiotic resistance of *Enteroccoci* species has also been recognized in other studies (da Costa et al. 2006, Kimiran-Erdem et al. 2007); the clinical difficulties in treating infections caused by such species are a result of this antibiotic resistance, warranting concern over their presence in the environment.

1.6 Occurrence of antibiotics in the aquatic environment

The occurrence of antibiotics in various water matrices has been studied to a significant extent, and world-wide reports of antibiotic occurrence are numerous. Various antibiotics have been identified in the wastewater of many treatment facilities as well as the waters receiving final wastewater effluent. Table 1.2 shows a comparison of concentrations of select antibiotics found in WWTP final effluents around the world.

In addition to contributions from wastewater, inputs from agricultural land have been identified as sources of contamination. Some studies have also addressed the issue of temporal and/or spatial effects on the occurrence of antibiotics. Antibiotic occurrence has been documented in many different countries in wastewater, surface water, groundwater, and even drinking water. A comprehensive overview of the voluminous amount of data is beyond the scope of this thesis. Therefore, this section will attempt to highlight, using representative studies, occurrence in different countries, with particular emphasis on North American studies. The occurrence in different aqueous environments including WWTPs, surface waters, ground waters and drinking waters will also be reviewed. The occurrence of some antibiotics with high potential for sorption has been noted in water sediments and wastewater sludges (see section 1.4.3) (Göbel et al. 2005b, Kim and Carlson 2007, Tolls 2001), but will not be a focus of this section. The information presented illustrates the impact that human and agricultural inputs of antibiotics have on environmental waters, and that this trend has been noted world-wide.

1.6.1 Occurrence in Canada

In 2004, the first Canadian study to look at a variety of antibiotic compounds in wastewater effluent was published (Miao et al. 2004). The final effluent from eight WWTPs in 5 Canadian cities was analyzed for the presence of a suite of antibiotics from the macrolide, sulfonamide, quinolone, tetracycline and quinoxaline dioxide classes. Of the antibiotics surveyed, ciprofloxacin, clarithromycin, erythromycin-H_2O (a degradation

Table 1.2: Comparison of antibiotic concentrations measured in WWTP final effluent samples world-wide

	Concentrations measured in final effluent (µg/L)			
Antibiotic	**Canada**	**United States**	**Europe**	**Asia**
Ciprofloxacin (fluoroquinolone)	0.118[a], 0.311[g]	0.091–5.6[b]	0.045–0.108[d]	—
Sulfamethoxazole (sulfonamide)	0.243[a], 0.29[f], 0.427[g]	0.37–6.0[b], 0.2[c]	0.352[e], 0.95–0.320[i]	0.136[k]
Trimethoprim	0.26[f], 0.094–0.344[h]	0.09–0.53[b], 0.17[c]	0.081[e], 0.012–0.180[i]	0.058[k]
Tetracycline (tetracycline)	0.151[a], 0.244[g]	0.14–0.56[b], 0.17[c]	—	—
Clarithromycin (macrolide)	0.087[a], 0.91[f]	—	0.220[e]	—
Erythromycin (macrolide)	0.080[a], 0.092[f], 0.030[g]	0.27[c]	0.055[e]	0.130[k]

a = (Miao et al. 2004); b = (Batt et al. 2006a); c = (Karthikeyan and Meyer 2006) d = (Golet et al. 2001); e = (Göbel et al. 2004); f = (McClure and Wong 2007); g = (Chen et al. 2006); h = (Hua et al. 2006); i = (Botitsi et al. 2007); k = (Kim et al. 2007)

product of erythromycin), ofloxacin, sulfamethoxazole, sulfapyridine, and tetracycline were the most frequently detected. Important conclusions from this study were that antimicrobials detected in wastewater effluents were those used for human medicine; compounds used exclusively for animals were not detected; and finally, the compounds present did not exceed concentrations of 1 µg/L, at least in that study. A glimpse into the identity and amounts of important human-use antibiotics entering the Canadian environment was afforded by this study, and will likely serve as a foundation upon which further investigation into antibiotic occurrence in Canadian waters is built.

Hao et al. (2006) investigated the impacts of both urban and agricultural inputs of 27 antibiotics and neutral pharmaceuticals to surface water in the Grand River watershed in Ontario. Seven of the eight sampling sites were chosen based on their high susceptibility to contamination by agricultural runoff, while the eighth site was chosen based on both urban and agricultural contributions to overall contamination. Antibiotics detected at the sites were: lincomycin, sulfamethazine, sulfachloropyridazine, trimethoprim, and erythromycin and were all in the ng/L concentration range. Lincomycin was the most prevalent of the antibiotics, and was found only at the sites receiving agricultural inputs. The authors noted that this was consistent with the types of agricultural activities occurring in the sampling areas. On the other hand, sulfachloropyridazine was found only at the urban input site (mean concentration of 20 ng/L) despite the fact that it is used in both human and veterinary medicine. It was, in fact, the only antibiotic detected at this site. The absence of this drug at agricultural impact sampling sites may reflect the relative amounts used in veterinary versus human medicine.

More recently, Lee et al. (2007) investigated the occurrence of the fluoroquinolone antibiotics ciprofloxacin, norfloxacin and ofloxacin in eight WWTPs in various cities in Ontario. Both primary and final effluents were analyzed for the presence of these compounds. In primary effluent, the median concentrations were 60 ng/L, 251 ng/L and 148 ng/L for norfloxacin, ciprofloxacin and ofloxacin, respectively. In final effluent, the median concentrations were reduced by more than 40% for both norfloxacin (34 ng/L) and ciprofloxacin (146 ng/L), while an increase (179 ng/L) was observed for ofloxacin. The authors hypothesized that this increase in concentration may be due to the deconjugation of ofloxacin metabolites during biological treatment of the wastewater. The results of this study are comparable to the 2004 study conducted by Miao et al. (2004), which was previously discussed. The previous study (Miao et al. 2004) also noted the presence of all three quinolones in the final effluents of various WWTPs across Canada occurring at median concentrations of 50 ng/L for norfloxacin, 118 ng/L for ciprofloxacin and 94 ng/L for ofloxacin. These values are comparable to the values obtained in the study by Lee et al. (2007), and reflect the similarities in occurrence in different parts of Canada.

The occurrence and temporal trends of selected pharmaceuticals and s-triazine herbicides were investigated in WWTP effluent and sites downstream on the upper Detroit River (Hua et al. 2006). The only antibiotic investigated in this work, trimethoprim, was found in all WWTP effluent samples in concentrations ranging from 92–435 ng/L. The concentration of trimethoprim was considerably higher in a sample collected in the summer versus a sample taken in the fall. Trimethoprim was also found at some of the downstream sampling sites, though in concentrations significantly lower than those found in the WWTP effluent. The farthest downstream site investigated was in the vicinity of raw water intake by the drinking water treatment plant for three Windsor, Ontario municipalities. In the March–April 2003 sampling period, trimethoprim was measured at this site. This clearly illustrates the potential for antibiotic drugs (amongst others) to reach drinking water.

A recent study conducted in Montreal, QC targeted six antibiotics (sulfamethoxazole, trimethoprim, ciprofloxacin, levofloxacin, clarithromycin, azithromycin) in wastewater influent and effluent (Segura et al. 2007). All targets were found to be present in both influent and effluent wastewater, with clarithromycin occurring at the highest concentration in two of three samples. Levofloxacin was found at the lowest concentrations in all samples. Estimated mean flows were calculated for all of the investigated compounds (g/day); the authors concluded that despite the low concentrations of antibiotics (µg/L) found in the WWTP effluent, large amounts of the compounds are discharged into the St. Lawrence River (between 118–830 grams/day for all compounds).

1.6.2 Occurrence in the US

In 2002, a comprehensive study by Kolpin et al. (2002) investigated the occurrence of a wide array of pharmaceuticals in the US aquatic environment. Several antibiotics were included in the list of target compounds, including numerous tetracylines, sulfonamides, quinolones, and some veterinary antibiotics. The studied bodies of water included 139 streams across 30 states; streams expected to be contaminated were favored in the selection of sampling sites. The thirty most frequently detected compounds included the antibiotics trimethoprim, erythromycin-H_2O, lincomycin and sulfamethoxazole. The authors noted that the absence of tetracycline and fluoroquinolone antibiotics was not unexpected given their propensity to sorb to sediments.

Batt and Aga (2005) investigated the occurrence of several antibiotics in groundwater contained in five different private drinking wells from Weiser, ID. Surface water from the Niagara River (Tonawanda, NY) and wastewater effluent from four WWTPs in different NY cities were also tested. None of the ground water samples, which were taken from wells located near a confined animal feeding operation, tested positive for any of the investigated compounds, which included sulfonamides, trimethoprim, fluoroquinolones, tetracycline, macrolides and the veterinary antibiotics lincomycin. A later study, however, confirmed the presence of two veterinary antibiotics, sulfamethazine and sulfadimethoxine, in private drinking wells in the same region (Batt et al. 2006c). Clindamycin, a lincosamide antibiotic, was detected in the surface water sample at a concentration of 1.1 µg/L. Two of the WWTP effluent samples, namely those from East Aurora and Holland, NY, were found to contain various concentrations (0.10–1.3 µg/L) of sulfamethoxazole, trimethoprim, ciprofloxacin, enrofloxacin and tetracycline. Sulfamethoxazole was found at the highest concentration in both WWTPs; a concentration of 1.3µg/L was found in both effluent samples.

The impact of antibiotics on the Cache la Poudre River in Colorado has been the subject of significant investigation (Yang and Carlson 2003, Yang et al. 2004). Owing to the diverse characteristics of this watershed, it was ideal for studying the occurrence of antibiotics in pristine, urban-impacted and agricultural-impacted aqueous environments. In a 2004 study conducted on the Cache la Poudre River, tetracycline and sulfonamide antibiotics were found to be present in three of the five river sites investigated (Yang et al. 2004). As expected, antibiotics were not found in the pristine river section (site 1) or just upstream of an urban area (site 2), indicating low agricultural and urban inputs. The remainder of the sites were located at points downstream of a WWTP, where various antibiotics were found. Samples from site 3, immediately downstream of the WWTP, contained the tetracyclines doxycline, tetracycline, chlortetracycline, oxytetracycline and the sulfonamide sulfamethoxazole. At site 4 (12 miles downstream) the tetracycline concentrations were found to increase or remain the same. An increase in tetracycline and oxytetracyline was seen at site 5, at which point agricultural land use was significant. Both of these compounds are approved as growth promoters for livestock. No doxycyline, which is a human use antibiotic, was found at site 5, indicating natural attenuation (e.g., sorption, photolysis, dilution) between sites 4 and 5. Sulfamethoxazole concentrations increased from sites 3 to 5 indicating further contributions between sampling sites. The sulfonamides sulfadimethoxine and sulfamethazine, both used in veterinary medicine, were first found at site 5, where agricultural influence is significant.

A recent study by Ye et al. (2007) revealed the presence of a number of antibiotics in finished, chlorinated drinking water from three drinking water treatment plants in North Carolina. Analysis of chlorinated drinking water samples was performed for 24 antibiotics from the sulfonamide, macrolide, fluoroquinolone, tetracycline and trimethoprim classes. Six antibiotics were identified in one or more of the samples: erythromycin, tylosin, roxithromycin, sulfamethoxazole, oxolinic acid, and flumequine. All were detected at mean concentrations below 5.0 ng/L. Corresponding measurements of drinking water treatment plant source waters confirmed the presence of all compounds found in finished drinking waters; the concentrations in source waters were higher, indicating some removal or transformation during treatment processes. For example, sulfamethoxazole and erythromycin were detected at 28 and 49 ng/L, respectively, in the source water of one of the water treatment plants

1.6.3 Occurrence in Europe

Reports from a number of European countries have indicated the presence of antibiotics in wastewaters, surface waters, and groundwaters.

The macrolide antibiotics erythromycin-H_2O, clarithromycin and roxithromycin were detected in three WWTP effluents in Switzerland (McArdell et al. 2003). Clarithromycin was the most abundant, and occurred at concentrations from 57–330 ng/L in the summer, in the various WWTP effluents investigated. Clarithromycin was detected in the receiving waters of the Glatt River at concentrations up to 75 ng/L, indicating incomplete elimination in WWTPs. Elimination of clarithromycin along a 36 km stretch of river was less than 20%. A later study by the same group measured antibiotics from the macrolide, sulfonamide and trimethoprim in primary, secondary, and tertiary wastewaters from two wastewater treatment plants, one of which was studied previously (Göbel et al. 2005b). Median concentrations of the detected compounds were 20–1700 ng/L for raw sewage and 10–400 ng/L in final effluent.

An analysis of 105 ground water samples in Baden-Württemberg, Germany revealed the presence of erythromycin-H_2O (maximum concentration 49 ng/L) and sulfamethoxazole (maximum concentration 410 ng/L) in 10 and 11 samples, respectively (Sacher et al. 2001). In a 1998 sampling, erythromycin, clarithromycin, roxithromycin, sulfamethoxazole and trimethoprim were found at concentrations between 30 and 70 ng/L in the river Elbe and its tributaries between the German-Czech Republic border by Wiegel et al. (2004).

Low levels of sulfamethoxazole (maximum concentration 10 ng/L) were found in Lake Maggiore in Italy; however it was not found in drinking water produced from the lake water (Loos et al. 2007). Sampling sites along the Po and Lambro Rivers in Italy were contaminated with various antibiotics including tylosin (0.29 ng/L), oxytetracycline (0.19–19.20 ng/L), oleadomycin (0.07–2.79 ng/L), lincomycin (3.13–248.90 ng/L), spiramycin (9.79–74.20 ng/L), erythromycin (1.40–15.90 ng/L), clarithromycin (0.49–20.30 ng/L) and ciprofloxacin (14.36–26.15 ng/L). Both rivers receive significant urban wastewater and agricultural inputs (Calamari et al. 2003).

Numerous studies from other European countries report similar findings of antibiotic compounds in wastewaters, surface waters and ground waters (Botitsi et al. 2007, Petrovic et al. 2006).

1.6.4 Occurrence in Asia

Recent reports on the occurrence of antibiotics in various water matrices have come from Asia (Kim et al. 2007, Xu et al. 2007b, Yasojima et al. 2006).

Yasojima et al. (2006) investigated the occurrence of levofloxacin (fluoroquinolone), clarithromycin (macrolide) and azithromycin (macrolide) in six WWTPs in Japan. Average concentrations were 557, 647 and 260 ng/L for levofloxacin, clarithromycin and azithromycin, respectively, in influent. Average removal of these compounds was calculated to be between 42–49%. An earlier study by Giger et al. (2003) found removal rates of between 80–90% for the fluoroquinolone antibiotics ciprofloxacin and norfloxacin during wastewater treatment. Göbel et al. (2005b) reported that no significant elimination of clarithromycin occurred in the primary and secondary treatment stages of a wastewater treatment plant, but that a 15% removal was achieved by sand filtration.

A survey of WWTP effluents, and various surface waters for the presence of various micropollutants in South Korea was conducted by Kim et al. (2007), and was, to the authors' knowledge, the first report of its kind for South Korea. Several antibiotics (erythromycin, sulfamethoxazole and trimethoprim) were present in effluent from seven

WWTPs at respective mean concentrations of 130, 136, and 58 ng/L. Surface water samples were also impacted by these antibiotics found in wastewater effluent, at respective mean concentrations of 3.4, 20, and 4 ng/L.

Sea water from Victoria Harbour (Hong Kong, China) and river water from Pearl River (Guangzhou, China) were collected and analyzed for the presence of nine antibiotics, in a study by Xu et al. (2007b). Selected sulfonamides, fluoroquinolones, and macrolides, as well as amoxicillin and chloramphenicol, were chosen as target analytes for the study. In Victoria Harbour, only some macrolides and quinolones were detected, indicating efficient removal of other compounds by water exchange between the harbor and the sea; the harbor treatment program previously implemented in the region was also thought to potentially remove some compounds. No seasonal differences in antibiotic concentrations were observed. Surface water samples from the Pearl River at Guangzhou were contaminated with antibiotics. Pearl River is the only receiving body of water for the estimated 1.7 million tones of wastewater discharged daily by the population of Guangzhou, which was estimated to be over 10 million in 2004. All of the investigated compounds, with the exception of amoxicillin, were found to be present in samples taken from the river in both high and low water seasons. Concentrations were much higher in the low water samples (median concentrations of 66–460 ng/L) versus the high water samples (median concentrations of 11–67 ng/L).

1.7 Analytical methods for the determination of antibiotics in water

In order to investigate the occurrence and fate of trace levels of antibiotics in complex aqueous samples, good extraction and analytical procedures are of great importance. The determination of low levels of antibiotic compounds in environmental aqueous samples has become possible with the development and improvement of analytical techniques. The extraction of antibiotics from aqueous environmental matrices is almost always performed using solid phase extraction, although other techniques such as solid phase microextraction, liquid-liquid extraction and lyophilization have been used to collect or concentrate antibiotics from these matrices (Fatta et al. 2007). In the following sections the SPE and SPME extraction techniques will be discussed in further detail.

The analysis of antibiotics is best carried out using reversed phase liquid chromatography with tandem mass spectrometric detection (MS/MS) using electrospray ionization (LC-ESI-MS/MS). The polar, non-volatile nature of many antibiotics makes them amenable to separation by reversed-phase liquid chromatography, while detection by MS/MS offers high sensitivity and the ability to unambiguously identify analytes of interest in complex matrices. Analysis has also been done by a number of other methods, which can include gas chromatography - mass spectrometry (GC-MS), LC-ultraviolet detection (Blackwell et al. 2004), and LC-fluorescence (Golet et al. 2001, Lee et al. 2007). The major drawback associated with the use of GC-MS is the need to derivatize polar compounds to more volatile forms in order to make them more amenable to analysis by GC. LC-UV techniques suffer from a lack of selectivity and/or sensitivity, which are two very important detector attributes when analyzing complex water samples that contain low levels of the target compounds. While LC-fluorescence is sensitive and selective, relatively few analytes fluoresce, limiting its utility. These techniques will not be addressed in significant detail in the following discussion. The focus of the following sections will instead be on the use of LC-ESI-MS/MS for the analysis of antibiotics in waste and environmental waters.

1.7.1 Extraction techniques

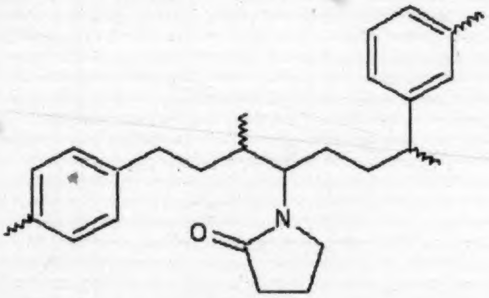
1.7.1.1 Solid phase extraction

Solid phase extraction (SPE) is the most widely used technique for the extraction of antibiotics from aqueous matrices (McArdell et al. 2006). A set volume of water is passed through a solid phase housed in a cartridge at a low enough flow rate to allow complete sorption of analytes (McArdell et al. 2006). The analytes of interest are retained as water is passed through the solid phase. The volume of water passed through the solid phase is dependent mainly upon the expected analyte concentration of the water sample. For example, lower water volumes (0.1–1 L) are used when extracting wastewater, versus the higher volumes required for extraction of surface waters (1 L or more).

Adjustment of sample pH and the addition of salts and chelating agents to the water samples can improve extraction recoveries of antibiotics. However, acceptable recoveries of analytes (>80%) can be obtained with little or no adjustment of sample pH/addition of salts and chelating agents, depending on the target analytes investigated and the choice of solid phase used for extraction (Gros et al. 2006, Nikolai et al. 2006, Weigel et al. 2004). To elute the analytes from the solid phase, a polar solvent such as methanol or acetonitrile is passed through the solid phase and the resulting analyte-containing eluate is collected. At this stage, the extract remains to be filtered, evaporated using nitrogen and amended with appropriate solvents and internal standards for analysis.

There are a wide variety of commercially available solid phases which are diverse in their characteristics, and therefore allow the selection of a solid phase most appropriate for the compounds of interest. Sorption of analytes to the solid phase can be due to a number of interactions (e.g., dispersion forces, hydrophobic interactions, ion exchange) depending on the composition of the solid material. Of the solid-phase extraction materials available for extraction of polar pharmaceuticals from water, the copolymer type solid phases are most often used. The Oasis HLB® solid phase is one such material, and is composed of a macroporous divinylbenzene-*N*-vinylpyrrolidone copolymer (Figure 1.3). Other commonly-used copolymer materials include polystyrene-divinylbenzene (e.g., LiChrolut® EN) and hyroxylated polystyrene-divinylbenzene (e.g., ENV+®) (Calamari et al. 2003, Lindberg et al. 2005). A survey of the literature reveals that the Oasis HLB is the most popular choice for the extraction of antibiotics from aqueous environmental matrices (Batt and Aga 2005, Göbel et al. 2004, Gros et al. 2006, Miao et al. 2004, Renew and Huang 2004, Yang et al. 2004). It offers a hydrophilic-lypophilic balance because of the two monomers that make up the copolymer; divinylbenzene imparts the lypophilic characteristics while vinylpyrrolidone gives the

Figure 1.3: Structure of divinylbenzene-*N*-vinylpyrrolidone copolymer
(Oasis HLB® SPE sorbent)



solid phase hydrophilic properties. This combination is very useful for the extraction of a wide array of compounds with different polarities.

Batt and Aga (2005) used the Oasis HLB for the simultaneous extraction of multiple classes of antibiotics (sulfonamides, trimethoprim, quinolones, macrolides, tetracyclines) from surface and groundwater. For the extraction of water samples, both the Oasis HLB and the tC_{18} Sep-Pak SPE cartridges were tested. The tC_{18} Sep-Pak cartridges were tested at a sample pH of 4 with addition of 0.1 M EDTA McIlvaine Buffer. The conditioning solvent was methanol, and eluent was methanol with 10 mM oxalic acid. The Oasis HLB cartridges were tested on samples with no pH adjustments and on samples adjusted to a pH of 3. Methanol and acetonitrile were tested as conditioning and eluent solvents. The addition of Na_2EDTA was also investigated in some of the protocols. The Oasis HLB gave more reproducible recoveries for the majority of the compounds investigated and subsequent extractions were carried out using these cartridges with the following conditions: addition of Na_2EDTA, sample pH of 3, and acetonitrile as the conditioning and eluent solvent. The addition of Na_2EDTA improved recoveries of the macrolide antibiotics investigated in the study. Adjustment of pH did not significantly affect recovery of any of the target analytes, with the exception of the fluoroquinolones, which had poor recoveries without a sample adjustment to pH 3. The study done by Miao et al. (2004) on occurrence of antibiotics in Canadian wastewater final effluents also demonstrated the effectiveness of the Oasis HLB sorbent. Using two different extraction protocols, good recoveries were obtained for all of the 31 antibiotic compounds considered in the study. It has been frequently observed that the addition of sodium EDTA to a water sample before extraction can improve the extraction recovery for tetracycline antibiotics. This was observed by Miao et al. (2004) in the study described previously. The authors found that this addition also improved the recoveries of the fluoroquinolones investigated in the study. Gros et al. (2006) developed a solid phase extraction method targeting the simultaneous extraction of various pharmaceuticals, including some antibiotics (trimethoprim, sulfamethoxazole, azithromycin, erythromycin, ofloxacin). Of the sorbents investigated, Oasis HLB gave the best performance and surface and wastewater extractions were successfully performed with no adjustment of sample pH. Many procedures exist in the literature targeting the extraction of individual antibiotic classes as well as multiple classes (Batt and Aga 2005, Golet et al. 2001, McArdell et al. 2003, Renew and Huang 2004).

SPE is an attractive extraction technique for a number of reasons. It is relatively straightforward to perform, multiple samples can be extracted simultaneously, and recoveries of target analytes are consistent. Additionally, extraction methods can be developed for the simultaneous extraction of diverse classes of compounds. Overall, the technique is quite simple and versatile, and is a suitable technique to precede analysis by LC-MS/MS.

Despite its versatility and widespread use, SPE of aqueous matrices does suffer from a number of limitations. While it is a relatively straightforward procedure to perform, sample preparation, extraction and processing is quite time-consuming. The large sample volumes required for each sample (0.1–1 L) mean that sample collection, transport and storage are a significant consideration. Perhaps the major drawback to SPE, however, is the tendency for coextraction of matrix components, including humic and fulvic acid, from the water samples onto the solid phase. This is particularly an issue with wastewater, in which a multitude of organic matrix components are present. After extraction is complete and elution of analytes is done with polar solvent, some of the coextracted material is eluted with the desired analytes, producing a very complex and "dirty" extract. The two major impacts of this coextraction of matrix are interference by the matrix components leading to reduced recoveries of target analytes, and interference in analysis by LC-ESI-MS/MS by the matrix components present in the final extract. The deleterious effects of wastewater derived extracts are frequently seen in analysis by electrospray mass spectrometry. The presence of these additional matrix interferences can affect the overall electrospray ionization process leading to either suppression or enhancement of analyte ionization. These processes may therefore affect the reproducibility and accuracy of measurements obtained using ESI-MS/MS.

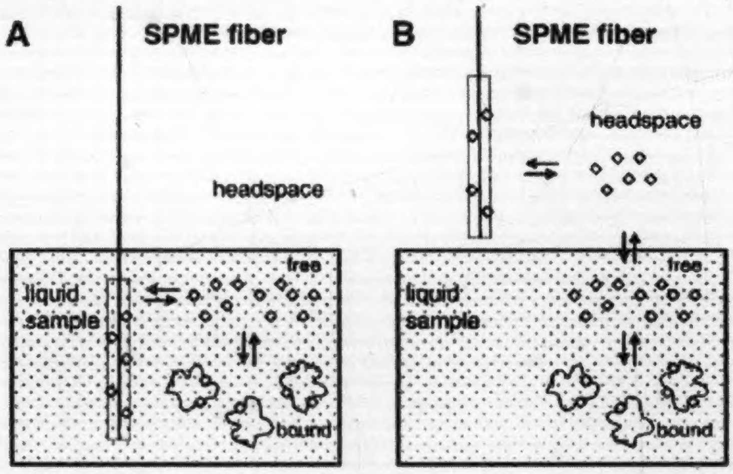
A number of strategies have been employed to correct or eliminate matrix effects in extracts. Sample clean-up before or after solid phase extraction using various material has been used with success (Balakrishnan et al. 2006, Renew and Huang 2004). However, additional extractions added to the process can lead to analyte losses, and increase the overall time required for sample preparation. Calibration-based approaches include internal standard calibration, and standard addition calibration. Dilution of extracts has also been done in order to decrease the concentration of matrix that is analyzed. Of all of the possible approaches used to correct for matrix effects, internal standard calibration tends to be the most popular. Its simplicity is attractive as it requires only the addition of one or more structurally similar or isotopically labeled compounds to calibration standards and sample extracts. It is generally not possible to find isotopically labeled versions of all target analytes, making it necessary to rely on a single internal standard (either isotopically labeled, or structurally similar and not present in the sample) for a number of different analytes. Although this method will correct, to some extent, for matrix effects, it has been shown that the effectiveness will vary from compound to compound (Göbel et al. 2004, Renew and Huang 2004). However, its simplicity and ability to correct for matrix effects makes it a good choice in approaching the issue of matrix effects in analysis. For those compounds where matrix effects are not sufficiently compensated for by the addition of internal standards, other approaches may be necessary.

The method of standard addition will correct for any matrix effects, but is unfortunately a very time consuming process when used with solid phase extraction. Good results have been obtained using standard addition, where water samples are amended with increasing amounts of target analytes, extracted, and the resulting calibration curve extrapolated to give the concentration of target analytes natively present in the sample (Botitsi et al. 2007). However, the time-consuming nature of this approach is a significant drawback, and it is used to a lesser extent than internal standard calibration.

1.7.1.2 Solid phase microextraction

Solid phase microextraction was introduced in the early 1990's by Arthur and Pawliszyn (1990) and has since found numerous applications in environmental chemistry (Koester 2005, Peñalver et al. 2002b, Quintana and Rodríguez 2006, Sarrion et al. 2000, Vas and Vékey 2004). The SPME apparatus consists of an optical fiber coated with a solid phase and is attached to a holder which controls the exposure of the fiber to solution or headspace. The fiber is exposed to the sample of interest for a predetermined period and then the sorbed analyte is removed either thermally in the injection port of a GC, or by using an appropriate solvent to desorb the analyte from the fiber.

Figure 1.4: Representation of (A) direct immersion solid phase microextraction and (B) headspace solid phase microextraction[1]



Because the volume of the extraction phase is very small compared with the sample volume, extraction of analytes is non-exhaustive, unlike SPE where, as described previously, exhaustive extraction of a given sample is performed. SPME can be used to extract analyte from headspace, for more volatile compounds, or can be directly immersed in an aqueous sample for compounds with low volatility, such as antibiotics (Figure 1.4). Partitioning occurs via adsorption or absorption, depending on the identity of the solid phase. Liquid-type phases, such as polydimethylsiloxane (PDMS), follow absorption behavior, while solid phases such as Carbowax Templated Resin (CW/TPR) and PDMS/divinylbenzene (PDMS/DVB) work by an adsorptive mechanism (Lord and Pawliszyn 2000b).

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[1] Reprinted with permission from Musteata, F. M. and Pawliszyn, J. (2007), "*In vivo* sampling with solid phase microextraction", *Journal of Biochemical and Biophysical Methods*, **70** 181-193, Copyright (2007), Elsevier.

Analyte partitioning to the fiber is dependent upon analyte affinity for the extraction phase, sample matrix characteristics, and the type of extraction phase used. Equilibrium conditions for a given extraction have been reached when further extraction time yields no additional analyte uptake by the fiber. For solid sorbents, such as those investigated in this thesis, partitioning occurs by adsorption and follows a Langmuir isotherm at sufficiently high concentrations of analyte (Lord and Pawliszyn 2000b). The equilibrium amount of analyte extracted by the fiber (n_f^∞) for an adsorptive mechanism is described by the following equation (Lord and Pawliszyn 2000b):

$$n_f^\infty = C_f^\infty V_f = \frac{KC_s^0 V_s V_f \left(C_{f\max} - C_f^\infty\right)}{V_s + KV_f \left(C_{f\max} - C_f^\infty\right)} \qquad \textbf{(3)}$$

where C_f^∞ is the equilibrium concentration of analyte on the fiber, V_f is the volume of the fiber, K is the analyte adsorption equilibrium constant, C_s^0 is the initial concentration of analyte in the sample, and $C_{f\max}$ is the maximum concentration of active sites on the fiber coating.

Adsorption is a competitive process, and since more than one compound with affinity for the fiber coating is expected to be present in most samples, the mechanism governing adsorption in this situation is more complicated. The amount of analyte *A* extracted at equilibrium in the presence of analyte *B* is described by the following (Lord and Pawliszyn 2000b):

$$n_{fA}^\infty = C_{fA}^\infty V_f = \frac{K_A C_A^0 V_s V_f \left(C_{f\max} - C_{fA}^\infty\right)}{\left(1 + K_B C_{sB}^\infty\right) V_s + K_A V_f \left(C_{f\max} - C_{fA}^\infty\right)} \qquad \textbf{(4)}$$

If the amount of competing compound *B* is sufficiently low, or if it has little affinity for the fiber coating, the terms for the competing compound may be insignificant, and extraction of compound *A* is not affected by the presence of additional compounds (Lord and Pawliszyn 2000b).

Analyte sorption to the fiber coating is also dependent upon the various phases present in a sample. Analyte partitioning to competing phases may occur, effectively reducing the amount extracted by the fiber (Lord and Pawliszyn 2000b). There are a number of parameters that can be manipulated to increase extraction efficiency in SPME. A brief description of these parameters, which include sample agitation, pH, extraction temperature, ionic strength and extraction and desorption time, are presented.

Sample agitation will increase the extraction speed by reducing the size of the boundary layer around the fiber coating. This may be accomplished by a stir bar or by other means, and ensures that as the analyte concentration in the immediate area around the extraction phase becomes diminished, more analyte is delivered to the fiber surface for sorption (Lord and Pawliszyn 2000b).

For analytes with acidic or basic functionalities, sample pH can be an important consideration in achieving the greatest extraction efficiency. For both absorptive and adsorptive type fiber coatings, it has been shown that an ionizable analyte is most readily extracted in its neutral form (Aresta et al. 2003, Aresta et al. 2005, Chou and Lee 2005). Therefore adjusting the sample pH may have a significant impact on the overall amount of analyte extracted.

Increasing the ionic strength of a solution has been shown to increase extraction efficiency of some analytes by SPME (Aresta et al. 2005, Balakrishnan et al. 2006, Lock et al. 1999, Lord and Pawliszyn 1997). Upon addition of a neutral salt, such as NaCl, hydration spheres form around the ionic salt molecules. This effectively shifts the equilibrium in favor of the solid phase and the overall amount of analyte extracted is increased. At increasing salt concentrations, however, polar analytes may begin to interact with the salt, leading to a decrease in extraction amount (Lord and Pawliszyn 2000b).

Temperature increases of the sample solution typically decrease the overall amount of analyte extracted (Aresta et al. 2005, Wu and Huang 1999), though increases in extraction efficiency with increasing temperature have been noted (Lamas et al. 2004, Lock et al. 1999). Two processes occur when solution temperature is increased: diffusion coefficients of analytes are increased, while partition coefficients are decreased. The increased diffusion coefficients result in a shorter time to reach equilibrium, while the decreased partition coefficients result in a decreased amount of analyte extracted at equilibrium (Lord and Pawliszyn 2000a).

Ideally, extractions should be carried out under equilibrium conditions as there will be no further changes in analyte uptake by the fiber after equilibrium conditions have been reached. This ensures that error in measurements is minimized. In some cases, the time to reach equilibrium is prohibitively long, therefore it becomes necessary to carry out extractions under non-equilibrium conditions. As long as all extraction parameters are kept identical from one extraction to another, good results can be achieved, even under non-equilibrium conditions (Lord and Pawliszyn 2000b).

Desorption of analytes off of the fiber should also be optimized. Whether the analytes are desorbed thermally or in solvent, this parameter should be optimized to ensure that there is no carry-over of analyte from one extraction to another.

Fairly limited work on the SPME of antibiotics and other pharmaceuticals from aqueous environmental matrices has been done to date (Balakrishnan et al. 2006, Hernández et al. 2007, Lamas et al. 2004, Moeder et al. 2000). However, SPME has been used extensively in the extraction of pharmaceutical compounds from biological matrices such as blood, serum and urine, so its application to these compounds in complex matrices has been proven (Lord and Pawliszyn 2000b) As an alternative to SPE for the extraction of antibiotics from waste and surface waters it possesses a number of desirable characteristics including: decreased sample volumes, fast and simple extractions, and decreased or no transfer of sample matrix to final extracts.

There are several commercially available fibers to choose from, depending on the intended application. Generally the polarity of the analytes of interest, along with the intended method of analysis (i.e., LC or GC), will dictate which fibers are investigated; polar analytes will be most readily extracted by polar SPME fibers while the opposite is true for non-polar analytes. In addition, some fibers intended for GC use are not compatible with solvents used in LC, so this is another consideration when selecting a fiber. In the case of antibiotics, which are relatively polar compounds, polar fiber coatings such as the CW/TPR, polyacrylate (PA), and PDMS/DVB coatings should be the first choice for this type of analysis.

A handful of studies investigating the extraction of antibiotics and pharmaceuticals from aqueous environmental matrices exist in the literature, but the use of SPME for these applications is very limited. Recently, Balakrishnan et al. (2006) investigated the extraction of several sulfonamide antibiotics from wastewater influent using SPME. The results of the study indicated that SPME is a potentially useful extraction technique. Excellent recoveries of target analytes from fortified wastewater were obtained, and advantages over SPE were noted. Upon inspection of the data, however, it is clear that the limits of detection for the method (9.04–55.3 μg/L for the investigated sulfonamide antibiotics) are not sufficiently low to allow for quantification of analytes. This is not an unexpected result as the concentrations of antibiotics found in wastewater are on the order of ng/L–μg/L and SPME is a non-exhaustive extraction technique.

Successful determination of pharmaceuticals in environmental waters has been done by SPME followed by analysis using GC-MS. Lamas et al. (2004) applied a SPME method to the determination of selective serotonin reuptake inhibitors in river and wastewater while Rodriguez et al. (2004) determined various non-steroidal anti-inflammatory drugs in wastewater using SPME. Moeder et al. (2000) developed a SPME method for the extraction of pharmaceuticals including ibuprofen, paracetamol, phenazone, and carbamazepine and successfully applied the method to ground and river water in Germany.

The potential of SPME for the extraction of pharmaceutical compounds has been clearly demonstrated by the few studies that exist on the subject. This area of research warrants further investigation, as studies of antibiotic and pharmaceutical occurrence are likely to continue at a substantial rate and alternative extraction techniques will be of interest. Additionally, all but one of the studies found on this subject used GC-MS for detection. Expansion of the SPME technique to analysis by LC-MS/MS may make it a more attractive extraction option since much of this type of analysis is performed with LC-MS/MS.

1.7.2 Liquid chromatography - tandem mass spectrometry

Determination of antibiotics and other trace polar contaminants in environmental matrices has necessitated the use of analytical instruments which are sufficiently sensitive to detect the low levels of these compounds present in the environment (Fatta et al. 2007, Gros et al. 2006, Hernández et al. 2007). A liquid chromatograph interfaced to a mass spectrometer (LC-MS) has proven to be a valuable tool for this type of analysis; it is by far the most frequently used analytical method for analysis of antibiotics in the environment (Fatta et al. 2007). A number of other separation and detection methods have been investigated for the quantification of antibiotics, however, with the exception of gas chromatography - mass spectrometry (GC-MS), none can achieve the sensitivity, selectivity and overall performance associated with LC-MS.

Many pharmaceuticals are polar, non-volatile molecules, and some are thermally labile, thus separation by gas chromatography requires derivatization of these compounds to more volatile, stable forms. Derivatization is time-consuming and often yields inconsistent results; derivatizing reagents, such as diazomethane, are toxic and unpleasant to handle. These factors limit the usefulness and practicality of gas chromatography as a separation technique for polar pharmaceuticals, though it is still used regularly for the analysis of certain pharmaceuticals such as lipid regulators (bezafibrate, gemfibrozil, fenofibrate) and non-steroidal anti-inflammatory drugs (ibuprofen, ketoprofen, naproxen, diclofenac) (Fatta et al. 2007). Its use for environmental analysis of antibiotics has become increasingly limited in comparison to the use of LC-MS for these purposes (Fatta et al. 2007).

Liquid chromatography (LC) is well suited for the separation of polar, non-volatile compounds such as antibiotics, and has been used extensively for analysis of these types of compounds in environmental matrices (Anderson et al. 2005, Batt and Aga 2005, Yang et al. 2005). Reversed-phase liquid chromatography is used when LC is interfaced to a mass spectrometer, as normal phase solvents are generally incompatible with the electrospray ionization process (Cai et al. 2007). The most common stationary phase used to separate antibiotics is C_{18}, while typical mobile phases include water, acetonitrile and methanol. The addition of volatile modifiers such as ammonium acetate, and formic acid to mobile phases is done to control the pH of the mobile phase, and also to improve ionization efficiencies in electrospray ionization (Hernández et al. 2007). Overall, LC is a versatile separation technique, and is well suited to the separation of moderately polar to polar antibiotics.

The requirements for a suitable method of detection for the determination of trace levels of drugs in environmental matrices include sensitivity, selectivity, and reliability, amongst others. Detection methods which have been interfaced to LC for the detection of antibiotics in environmental matrices include: diode array (Babic et al. 2006), ultraviolet-diode array (Benito-Peña et al. 2006), fluorescence (Golet et al. 2001, Lee et al. 2007), and mass spectrometric detection. The spectroscopic detection techniques do not achieve the sensitivity or selectivity that is realized with mass spectrometric detection. Recently Lee et al. (2007) investigated the occurrence of fluoroquinolone antibiotics in wastewater using both fluorescence detection and mass spectrometric detection. Tandem mass spectrometry with electrospray ionization was found to be superior in terms of sensitivity and selectivity.

Detection of antibiotics in environmental extracts by mass spectrometry is usually accomplished by using a single quadrupole (MS) or triple quadrupole mass spectrometer (MS/MS) with an electrospray ionization source. Quadrupole mass spectrometers are the most widely used mass analyzer, owing to their simplicity, low cost, and performance (Niessen 1999). Analysis by triple quadrupole instruments is a popular way to perform tandem mass spectrometry (MS/MS). The triple quadrupole instrument consists of three quadrupoles in series and analysis utilizing all three quadrupoles (MS/MS) results in high sensitivity, selectivity and unambiguous identification of target analytes (Niessen 1999). These attributes are maximized by the use of the instrument in various scanning modes. For environmental analysis, multiple reaction monitoring (MRM) mode is the most important. In this mode, precursor ions resulting from the ionized analyte molecules are selectively passed through the first quadrupole, while all other mass to charge (m/z) values are filtered out. Once the ion of interest has passed through the first quadrupole, it is fragmented in the second quadrupole, which functions as a collision cell. The most abundant fragment is then passed through the third quadrupole, where it is detected. This process results in very high sensitivity, since only the mass to charge ratios of interest are detected, while everything else is filtered out. The selectivity is also very high, and unambiguous identification of target analytes in complex environmental samples can be made based on the unique combination of mass to charge ratios of precursor and product ion(s).

The atmospheric pressure ionization technique of electrospray ionization (ESI) is typically used to generate ions for detection by MS when coupled with LC. This technique is well suited for the analysis of polar, thermally sensitive compounds, and is considered a soft ionization technique, since fragmentation of molecules is limited. In the ESI process, gas phase ions are produced by passing the analyte-containing LC mobile phase through a high voltage capillary. The sample is nebulized to charged droplets during this process, and continual evaporation of solvent from the solute molecules leads to the production of gas phase analyte ions. Typically for small molecules, singly charged species are produced. In the positive ionization mode, a positive voltage is applied to the capillary, and protonated gas phase ions are produced, while in negative mode, the analyte is deprotonated. The functional groups present in the analyte molecule of interest dictate the appropriate ionization mode. Molecules containing acidic functionalities will be more readily analyzed in negative mode, while those containing basic functionalities will be determined in positive mode. The ions are introduced to the mass analyzer, where they are then sorted based on their mass to charge ratio (*m/z*), as described previously.

1.7.2.1 Ionization suppression

Electrospray ionization is highly susceptible to the presence of additional matrix components in environmental extracts produced by SPE. A thorough discussion of the matrix effects observed in analysis of aqueous environmental SPE extracts by LC-ESI-MS/MS was presented previously (section 1.7.1.1). Sample preparation and calibration-based approaches for the reduction/correction of matrix effects were also discussed. The present discussion will be limited to a brief description of potential mechanisms of the ion suppression/enhancement often observed with the ESI source, and the possible approaches for overcoming these effects at the instrumental level.

Since the introduction of LC-MS with atmospheric pressure ionization (API) as a suitable technique for the analysis of polar compounds, the main shortcoming that has been identified is the presence of so-called "matrix effects", with ion suppression being the predominant effect observed (Antignac et al. 2005). Clearly any variation in ionization efficiency can lead to undesirable effects on analyte signal, thereby reducing method performance with regard to reproducibility, sensitivity, precision and accuracy.

The complexity of extracts generated by SPE, particularly for a matrix like wastewater, makes these types of analyses prime candidates for ion suppression problems. After chromatographic separation, analytes and coeluting compounds are passed into the electrospray source where ionization takes place. The greatest ion suppression is expected to occur when the coeluting compound has a high mass, high basicity and high concentration (Jessome and Volmer 2006). There are several proposed mechanisms by which ionization suppression occurs. One proposed mechanism hypothesizes that high concentrations of interfering compounds lead to a decrease in droplet evaporation efficiency, ultimately interfering with the successful transfer of target analytes to the gas phase (Antignac et al. 2005). Another possible mechanism explains ion suppression as a function of saturation at the surface of ESI droplets when high concentrations ($>10^{-5}$ M) are present. Competition for surface space or charge by analytes with different characteristics will lead to various extents of signal suppression for individual constituents when dealing with high concentration, multicomponent samples (Jessome and Volmer 2006).

Numerous strategies have been suggested to overcome ionization suppression at an instrumental level. Performing analyses in the negative ionization mode in ESI may reduce ionization suppression effects on target analytes. Fewer compounds are ionizable in negative ionization mode compared to positive mode, and therefore less ionization suppression may be seen in this mode (Jessome and Volmer 2006). This approach would only be suitable for target analytes which give a signal in negative mode. Source geometry has been found to affect the extent of ionization suppression; a study by Souverain et al. (2004) determined the ionization effects to increase in the order: Z-Spray, orthogonal spray, linear spray geometries. Switching to an alternative source, such as atmospheric chemical ionization, or atmospheric pressure photoionization (APPI) may help reduce matrix effects as these modes of ionization may be less susceptible to ionization suppression.

Modifying the chromatographic conditions has also been suggested as an effective way to deal with ionization suppression. The chromatographic regions expected to be most affected by ionization suppression are the solvent front, where unretained compounds are eluted, and the gradient end, where strongly retained compounds will be eluted (Antignac et al. 2005, Jessome and Volmer 2006). Modifying chromatographic conditions to ensure elution of target analytes between these two problem areas is one relatively simple approach to reducing the impact of ionization suppression on analytes of interest (Antignac et al. 2005, Jessome and Volmer 2006).

Clearly there are a number of different approaches to dealing with the issue of ionization suppression in ESI-MS. Jessome and Volmer (2006) suggest that the most effective approaches to circumventing the issue are to improve sample preparation and adjust chromatographic conditions.

2 Analytical methods development and environmental measurements[2]

2.1 Introduction

The issue of antibiotics and other pharmaceuticals as emerging environmental contaminants has been an increasing focus of recent environmental research (Daughton and Ternes 1999, Halling-Sørensen et al. 1998, Richardson and Ternes 2005). Significant and continual inputs of antibiotics to the environment occur via disposal of expired or unused drugs and by human and animal excretion. Some of the major concerns about this continual input to the environment include possible chronic effects to non-target organisms (Fent et al. 2006), and the development of antibiotic-resistant microbes (Davison 1999, Xu et al. 2007a). Research aiming to understand the occurrence, fate and effects of these compounds in the environment is underway, but presently knowledge in these areas is incomplete (Richardson and Ternes 2005).

To understand the behavior of antibiotics in the environment, reliable and sensitive measurement methods must first be established. Antibiotics have frequently been detected in the ng/L to µg/L concentration range in wastewaters and at ng/L concentrations in surface waters, (Calamari et al. 2003, Castiglioni et al. 2005, Golet et al. 2001, Kolpin et al. 2002, Lindberg et al. 2005, McArdell et al. 2003, Miao et al. 2004) and methodology for both the extraction and analysis of many antibiotics from these water matrices is becoming well established in the literature (Anderson et al. 2005, Batt and Aga 2005, Renew and Huang 2004, Ternes et al. 2001, Yang et al. 2005). Typically, the extraction of antibiotics from waste and environmental waters is accomplished using solid phase extraction (SPE), and analysis is performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI). This type of analytical procedure has proven effective in evaluating the trace concentrations of antibiotics present in complex water matrices. Processing by SPE allows simultaneous extraction of multiple samples and generally gives good recovery of target compounds (Miao et al. 2004), while analysis by electrospray LC-MS/MS allows for high selectivity and sensitivity (Ternes 2001, Vanderford et al. 2003). As such, these techniques are well suited for the analysis of antibiotics in the environment.

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Despite the widespread use of SPE for the extraction of pharmaceuticals from environmental waters, there are several disadvantages associated with its use for these applications. First, SPE is typically a laborious and time consuming process due to the large sample volumes needed (100 to 1000 mL per sample) (Hao et al. 2006, Miao et al. 2004, Nikolai et al. 2006) to provide measurable signals for electrospray LC-MS/MS analysis, and the multiple steps involved in processing the extracts. In addition, a significant disadvantage of SPE is the coextraction of unwanted matrix components from complex matrices such as wastewater. The additional matrix components present in extracts are generally at much higher concentrations than the analytes of interest, and can result in significant ESI ion suppression that confounds quantification (Benijts et al. 2004, Lindberg et al. 2004, Nikolai et al. 2006, Petrovic et al. 2005, Richardson and Ternes 2005).

The use of solid phase microextraction (SPME) for the extraction of pharmaceuticals from water is of interest, as it may offer benefits over traditional SPE techniques. The SPME apparatus consists of a coated optical fiber attached to a holder that guides, protects and controls the exposure of the fiber. The fiber coating is selected based on the intended application, and is composed of a solid, liquid or a combination of the two. Upon exposure to a sample, sorption of compounds to the fiber occurs. The nature of SPME is such that the extraction, clean-up and concentration of a sample occur simultaneously, thus greatly reducing required time and labor (Vas and Vékey 2004). In addition, SPME is potentially more cost-effective than SPE since an individual fiber can be used for multiple extractions (Vas and Vékey 2004) and very little solvent is required for the overall process. In contrast, SPE supplies are one-time use only and significantly more solvent is consumed. Another appealing benefit of SPME is the potential for overcoming the matrix effects often observed in analysis of SPE extracts. Unlike SPE, relatively little of the sample matrix components are transferred to final extracts, resulting in less interference in mass spectrometric analysis (Canosa et al. 2006).

Since its introduction in 1990 (Arthur and Pawliszyn 1990), SPME has found numerous applications in environmental chemistry, particularly for persistent organic pollutants (Koester 2005, Quintana and Rodríguez 2006, Vas and Vékey 2004). A wide range of other compounds including endocrine disruptors (Peñalver et al. 2002a), disinfection by-products (Sarrion et al. 2000), and phenolic compounds (Peñalver et al. 2002b), have been determined in various environmental matrices by SPME. The use of SPME for the extraction of pharmaceuticals has been investigated primarily in biological matrices such as milk, urine and plasma (Lord and Pawliszyn 2000b). Its application to environmental matrices is considerably less frequent, but has been accomplished in several studies. For example, Lamas et al. (2004) applied a SPME method to the determination of selective serotonin reuptake inhibitors in river and wastewater while

Rodriguez et al. (2004) determined various non-steroidal anti-inflammatory drugs in wastewater using SPME. Moeder et al. (2000) developed a SPME method for the extraction of pharmaceuticals including ibuprofen, paracetamol, phenazone, and carbamazepine and successfully applied the method to ground and river water in Germany. Analysis in these studies (Lamas et al. 2004, Moeder et al. 2000, Rodríguez et al. 2004) was performed using gas chromatography (GC). However, the polar, non-volatile nature of many such pharmaceuticals requires that derivatization be performed prior to analysis by GC. This process not only increases sample preparation times, but also can produce variable and/or incomplete derivatization of analytes (Ternes 2001). For these reasons, among others, LC is the preferred method for analysis of antibiotics and other pharmaceuticals. Balakrishnan et al. (2006) recently reported a SPME method for the extraction of sulfonamide antibiotics spiked into wastewater. Unlike previous studies, LC-MS/MS was used for analysis, thus eliminating the need for a derivatization step.

This work presents the development of a SPME method for the simultaneous extraction of several macrolide, sulfonamide and trimethoprim antibiotics from wastewater matrices. The target compounds have been frequently detected in environmental waters (Batt and Aga 2005, Kolpin et al. 2002, Miao et al. 2004). We apply this method to wastewater samples taken from Gold Bar Wastewater Treatment Plant in Edmonton, Alberta. To our knowledge, this study is the first report using SPME to measure native concentrations of macrolides and trimethoprim in wastewater. We also compare the SPME-derived measurements to those obtained using SPE to assess the advantages and limitations of our SPME method.

2.2 Experimental

2.2.1 Reagents and standards

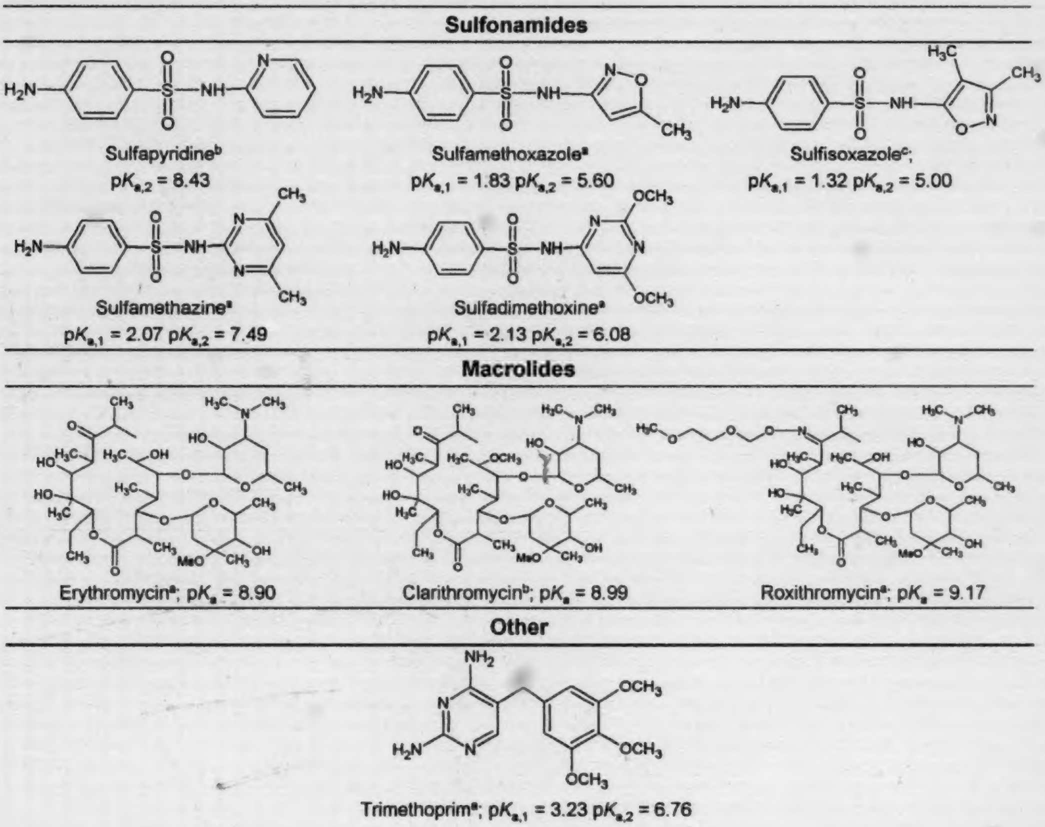
Sulfamethazine, sulfisoxazole, sulfamethoxazole, sulfadimethoxine, sulfapyridine, trimethoprim, roxithromycin, and erythromycin were purchased from Sigma-Aldrich (Oakville, Canada). Clarithromycin was purchased from Toronto Research Chemicals (North York, Canada) and josamycin from MP Biomedicals (Irvine, CA, USA). $^{13}C_6$-Sulfamethazine was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All standards, with the exception of sulfamethoxazole, clarithromycin, $^{13}C_6$-sulfamethazine (90%), and josamycin, were of 95% purity or greater. Analyte structures are shown in Figure 2.1. Standard solutions of antibiotics at a concentration of approximately 100 mg/L were prepared in methanol and stored in amber vials at 4°C. The standards were brought to room temperature before use and remade every three months; solution stability has been shown to be acceptable over this time period (Lindberg et al. 2004). Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Ottawa, Canada). A Nanopure Ultrapure Water System (Barnsteam/Thermolyne, Dubuque, IA, USA) provided nanopure (18 MΩ) water used in experiments. Ammonium acetate (HPLC grade, 98.9% purity) and formic acid (99% purity) were purchased from Fisher. All reagents were used as received.

2.2.2 Sample collection and preparation

Grab and composite (24 hour) influent and final effluent wastewater samples for method evaluation and quantification were collected from Edmonton Gold Bar Wastewater Treatment Plant, a tertiary treatment plant servi a population of approximately 700,000, on May 14, 2007. Samples were stored in 4 L amber glass bottles

Figure 2.1: Target analyte structures and corresponding pK_a values

The $pK_{a,1}$ and $pK_{a,2}$ values for the sulfonamide antibiotics correspond, respectively, to the amine (-NH_2) and amide (-NH-) moieties. References: superscripted a = Qiang and Adams, 2004; b = Wishart et al. 2006; c = Geiser et al. 2005.



Sulfonamides
Sulfapyridine[b]
$pK_{a,2}$ = 8.43
Sulfamethoxazole[a]
$pK_{a,1}$ = 1.83 $pK_{a,2}$ = 5.60
Sulfisoxazole[c];
$pK_{a,1}$ = 1.32 $pK_{a,2}$ = 5.00
Sulfamethazine[a]
$pK_{a,1}$ = 2.07 $pK_{a,2}$ = 7.49
Sulfadimethoxine[a]
$pK_{a,1}$ = 2.13 $pK_{a,2}$ = 6.08
Macrolides
Erythromycin[a]; pK_a = 8.90
Clarithromycin[b]; pK_a = 8.99
Roxithromycin[a]; pK_a = 9.17
Other
Trimethoprim[a]; $pK_{a,1}$ = 3.23 $pK_{a,2}$ = 6.76

and transported in ice-filled coolers from the collection site to the laboratory where they were then stored at 4°C until filtration. To remove particulates, filtration of the wastewater was performed the same day as sampling using 1.6 and 0.7 μm glass fiber filters precombusted at 450°C for 4 hours. Samples were processed within 3 days. The stability of antibiotics and other pharmaceuticals over this time frame has been previously demonstrated (Hao et al. 2006).

2.2.3 Water extraction: solid phase extraction

Oasis HLB cartridges (3 mL, 60 mg, Waters, Mississauga, Canada) were used for the solid-phase extraction of target analytes from the wastewater samples. After conditioning each cartridge with 2×3 mL of methanol and 2×3 mL of nanopure water, polytetrafluoroethylene (PTFE) sippers (Scientific Products & Equipment, North York, Canada) were used to deliver 100 mL influent samples and 500 mL effluent samples at 2 mL/min. No pH adjustments were made to the wastewater samples, as Oasis HLB cartridges have been shown to be effective at neutral pH for collection of antibiotics from environmental waters (Batt and Aga 2005). After extraction, elution of each cartridge was performed with 6 mL of methanol. The eluent was subsequently passed through a sodium sulfate drying column, filtered through a 0.22 μm PTFE syringe filter, and evaporated to dryness using a nitrogen evaporator. Extracts were reconstituted with methanol and fortified with the internal standards $^{13}C_6$-sulfamethazine and josamycin to a final volume of 1 mL.

Quantitation of analytes was done using internal standard calibration (Table 2.1). Calibration standards were prepared in methanol at concentration levels of 1–100 μg/L and internal standards were added to all standards and samples. To evaluate the precision of the method, all samples were done in triplicate. Recovery samples spiked with 100 ng of each target analyte were used to estimate extraction recoveries for both influent and effluent samples. Recoveries were calculated by subtracting native concentrations determined from unspiked samples from concentrations of recovery samples. Procedural blanks were processed with each set of samples to monitor for any possible contamination with target analytes.

2.2.4 Water extraction: solid phase microextraction

Several commercially available SPME fiber assemblies were investigated for the extraction of the target antibiotics from aqueous solution: carbowax/divinylbenzene (CW/DVB, film thickness 65 μm), polydimethylsiloxane (PDMS, 100 μm), polydimethylsiloxane/divinylbenzene (PDMS/DVB, 60 μm) and Carbowax-templated resin (CW/TPR, 50 μm) (Supelco, Oakville, Canada). Direct immersion extractions were performed, with stirring, on 1.5 mL of sample contained in glass amber vials. The fiber was immersed in the sample for extraction. This was followed by desorption of analytes off the fiber, by immersing it in 1.5 mL of stirred methanol. Optimum extraction and desorption times were 30 and 10 minutes, respectively, as described in detail in the results and discussion section. The resulting extracts were evaporated to dryness using a nitrogen evaporator, then reconstituted with 30% acetonitrile/70% water and internal standard solution to a final volume of 75 μL. Quantitation of analytes was accomplished using standard addition curves generated by SPME extractions of wastewater spiked with increasing amounts of target analytes; spike levels were 0 to 5000 ng/L.

2.2.5 Instrumental analysis

Analysis of samples was done with an Agilent 1100 HPLC and a Sciex QTrap 2000 triple quadrupole tandem mass spectrometer equipped with a TurboIonSpray ESI source (Applied Biosystems, Foster City, CA, USA). Analyses were done in positive-ion mode using multiple reaction monitoring (MRM). A ZORBAX Eclipse XDB C_{18} (4.6 mm × 250 mm I.D., particle size 5 μm, Agilent, Mississauga, Canada) or Ultra C_{18} (4.6 mm × 250 mm I.D., particle size 5 μm, Restek, Bellefonte, PA, USA) reversed-phase column was used to separate the target analytes using a gradient elution program and a 0.5 mL/min flow rate. Separations achieved by the two columns were very similar.

Acetonitrile and 10 mM ammonium acetate/0.1% formic acid/10% acetonitrile were used as the organic and aqueous mobile phases, respectively. The gradient was increased from 28% to 55% acetonitrile over 11 minutes and then increased to 95% acetonitrile over the next two minutes. This was followed by a seven minute equilibration time for a total runtime of 20 minutes. Injection volumes of 10 and 50 µL were used for SPE and SPME experiments, respectively. The larger volume chosen for injection of SPME extracts (50 µL) had no undesired effects on chromatography, based on tests performed with varying injection volumes. Analyst® 1.4.1 software (Applied Biosystems, Foster City, CA, USA) was used for instrument control, data acquisition, and data processing. Tandem mass spectrometry parameters were optimized for the target analytes: curtain gas setting of 35; collisionally activated dissociation (CAD) gas setting of 4; nebulizing gas and heater gas settings of 45 and 90, respectively; heater gas temperature of 500°C; ion energy 5500 V. Nitrogen was used as curtain gas and CAD gas, while zero air was used as nebulizing gas and heater gas. Ionization parameters optimized for individual compounds are summarized in Table 2.1.

To account for instrumental variability and matrix effects, internal standards were used in all SPE extracts. Standard addition was used to quantify analytes in SPME extracts. However, internal standards were added to all SPME extracts to assess resulting matrix effects in analysis as described in detail in the results section. $^{13}C_6$-Sulfamethazine was used as an internal standard for the sulfonamide and trimethoprim classes of antibiotics, while josamycin was used as the internal standards for the macrolide antibiotics. Unspiked wastewater samples were analyzed for the presence of josamycin, as it is not isotopically labeled. No josamycin was found.

Table 2.1: Optimal LC-ESI-MS/MS parameters used in this study
For target analytes in positive-ion mode with multiple reaction monitoring (MRM). [a]*internal standard for analyte class.* [b]*Internal standard used was* $^{13}C_6$*-sulfamethazine.*

Compound	MRM transition	Collision energy (eV)	Declustering Potential (V)	Cell exit potential (V)	Cell entrance potential (V)
Sulfonamides					
$^{13}C_6$-Sulfamethazine[a]	285>124	30	65	10	3
Sulfapyridine	250>156	20	60	10	3
Sulfamethoxazole	254>156	20	65	10	3
Sulfisoxazole	268>156	20	80	10	3
Sulfamethazine	279>186	20	65	10	3
Sulfadimethoxine	311>156	25	80	10	3
Macrolides					
Josamycin[a]	828.5>109	65	100	10	2
Erythromycin	734.5>158	40	100	10	2
Clarithromycin	748.5>158	37	100	10	3
Roxithromycin	837.5>158	45	100	10	3
Other					
Trimethoprim[b]	291>230	30	80	10	3

A large portion of erythromycin present in wastewater has been shown to occur as its degradation product anhydroerythromycin (erthyromycin-H_2O) (Kolpin et al. 2002, McArdell et al. 2003, Miao et al. 2004, Vanderford et al. 2003). For this reason, many researchers detect and quantify erythromycin-H_2O instead of the parent compound. In the present study, detection and quantification was done for erythromycin, and not its degradation product. The reason for this was two-fold: first, erythromycin was detected in the wastewater samples investigated in this work, and second, no standard is currently available for anhydroerythromycin. Erythromycin-H_2O may behave in a manner that differs from erythromycin in analysis by LC-MS/MS, suggesting that the use of an erythromycin standard to quantify this degradation product is inappropriate.

2.3 Results and Discussion

2.3.1 Optimization of SPME parameters

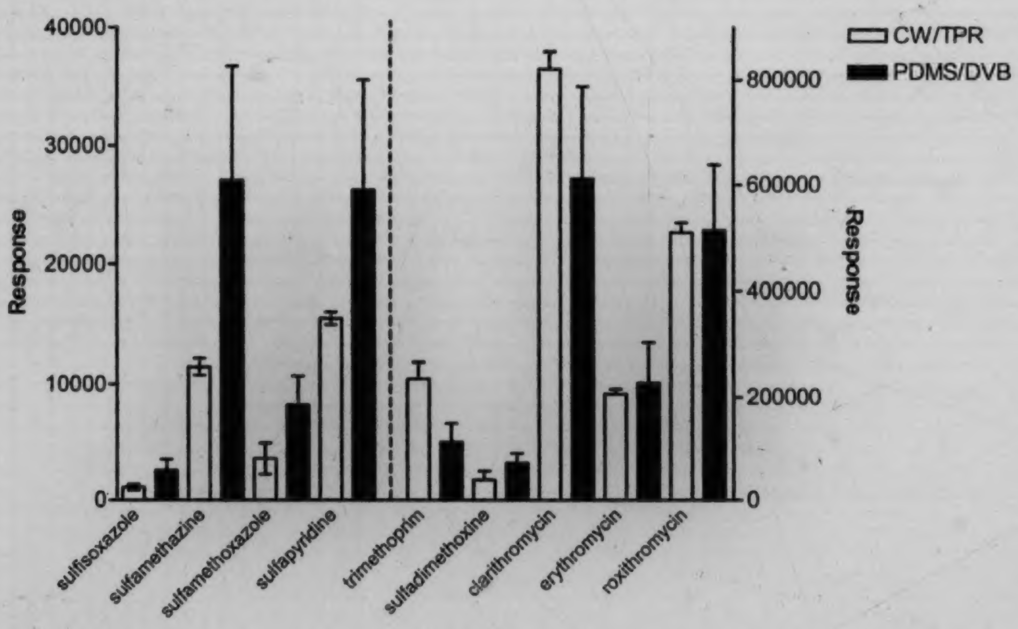
In order to maximize extraction efficiency of target analytes, several parameters were optimized for the SPME method. Experiments were carried out to determine optimal fiber type, extraction and desorption time, pH, ionic strength, and extraction temperature. Our aim was to find conditions suitable for SPME extraction of multiple classes of antibiotics, rather than individual classes of analytes, as the former is more representative of actual use and occurrence. Thus, the optimal conditions described in this study may not necessarily be optimal for specific compounds, but would accommodate the extraction of the greatest number of target compounds possible under a given set of conditions. However, the effect of individual parameters on extraction efficiency is noted in this study, so that optimizations for individual compounds or classes can be done.

2.3.2 Fiber selection

Several commercially available fiber assemblies were considered for use in this work: 65 µm CW/DVB, 50 µm CW/TPR, 100 µm PDMS, and 60 µm PDMS/DVB. Extractions were performed on stirred 100 µg/L solutions of target compounds for 30 minutes followed by a 10 minute desorption into methanol. The CW/DVB is a fiber intended for GC applications and was incompatible with HPLC solvents; swelling of the stationary phase caused stripping of the fiber. Polar analytes, such as those under consideration, should have a greater affinity for a polar stationary phase. Accordingly, the polar CW/TPR and PDMS/DVB fibers were both able to extract the target analytes, while the non-polar PDMS fiber was ineffective (Figure 2.2).

Overall, the performance of the CW/TPR and PDMS/DVB fibers was comparable, with no statistical differences between compound responses on the two fibers (paired *t*-test, P>0.05). For several analytes, extractions performed with the CW/TPR fiber resulted in a more reproducible response, as indicated by the decreased error associated with measurements of these compounds (Figure 2.2).

Figure 2.2: Extraction efficiency of 50 µm CW/TPR and 60 µm PDMS/DVB SPME fibers
Left y-axis corresponds to analytes to the left of dashed line; right y-axis corresponds to analytes to the right of the dashed line. Error bars represent standard error of the mean (n=3 for each data point).

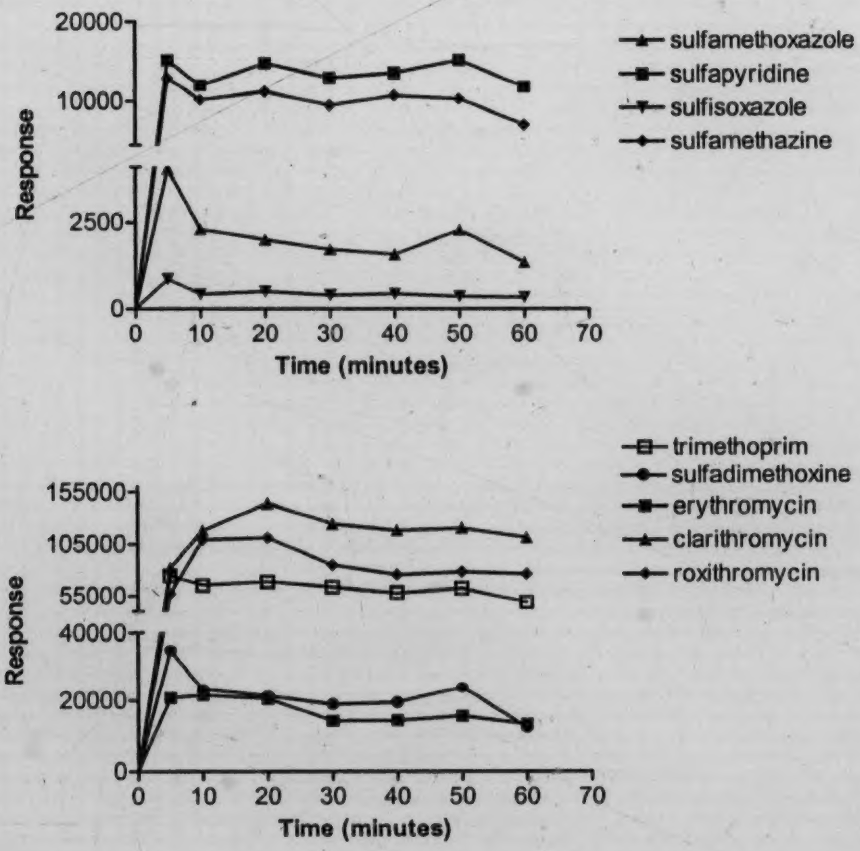


The CW/TPR fiber has performed acceptably in similar applications (Aresta et al. 2005, Balakrishnan et al. 2006, Lock et al. 1999). Both Lock et al. (1999) and Balakrishnan et al. (2006) favored the use of the CW/TPR fiber over the PDMS/DVB fiber for their respective work on extraction of tetracyclines from milk and extraction of sulfonamide antibiotics from wastewater. Based on its excellent performance, and use in similar applications, we chose the CW/TPR fiber for further investigation.

2.3.3 Extraction and desorption time

To study the effects of extraction time, 100 μg/L analyte solutions were extracted for various time periods ranging from 5 to 60 minutes. Desorption time was kept constant at 10 minutes per extraction. For the sulfonamides and trimethoprim, no significant increase in extraction mass was noted after approximately 10 to 20 minutes. The

Figure 2.3: Extraction time profile of target analytes (n=1)

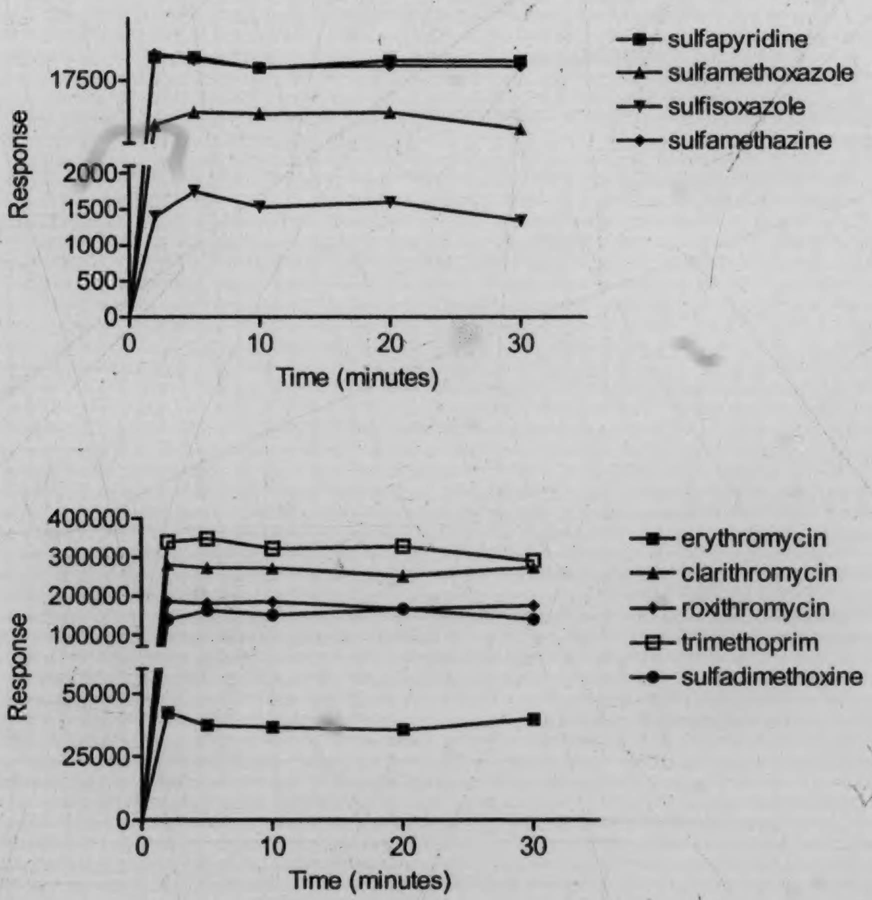


macrolides required a slightly longer extraction time in order to achieve maximum uptake. For these compounds, maximum extraction was observed by about 30 minutes (Figure 2.3). The extraction times are consistent with those reported in other studies that investigated the extraction of polar molecules using the CW/TPR fiber (Balakrishnan et al. 2006, Lock et al. 1999, Volmer and Hui 1997, Wu and Huang 1999). In their investigation of SPME of corticosteroids from urine, Volmer and Hui (1997) found that the time to reach maximum uptake of all compounds studied was between 10 and 60 minutes under stirred conditions with the addition of 25% NaCl. The authors chose an extraction time of 15 minutes, as 75% or more of the maximum concentrations were reached for target compounds within this time frame. Balakrishnan et al. (2006) noted a maximum uptake of sulfonamide antibiotics on a CW/TPR fiber within 20 minutes, consistent with the extraction profile of the sulfonamide antibiotics considered in our study. Based on our experiments, and the CW/TPR extraction profiles of other polar analytes, an extraction time of 30 minutes was chosen for further consideration. This extraction time allows for timely extraction of wastewater samples, and ensures that extractions are carried out under equilibrium conditions for all of the compounds, thereby avoiding error associated with non-equilibrium extraction (Lord and Pawliszyn 1997).

Using the same solution conditions, extractions were performed for 30 minutes with varying desorption times (2 to 30 minutes) to determine the optimal time for analyte desorption off the fiber (Figure 2.4). Desorption was rapid for all compounds, with complete desorption achieved in 5 minutes or less. This is consistent with other studies where relatively short desorption times were suitable (Volmer and Hui 1997, Wu and

Huang 1999). To ensure complete desorption and to mitigate any potential carry-over effects, a desorption period of 10 minutes was chosen.

Figure 2.4: Desorption time profile of target analytes (n=1)

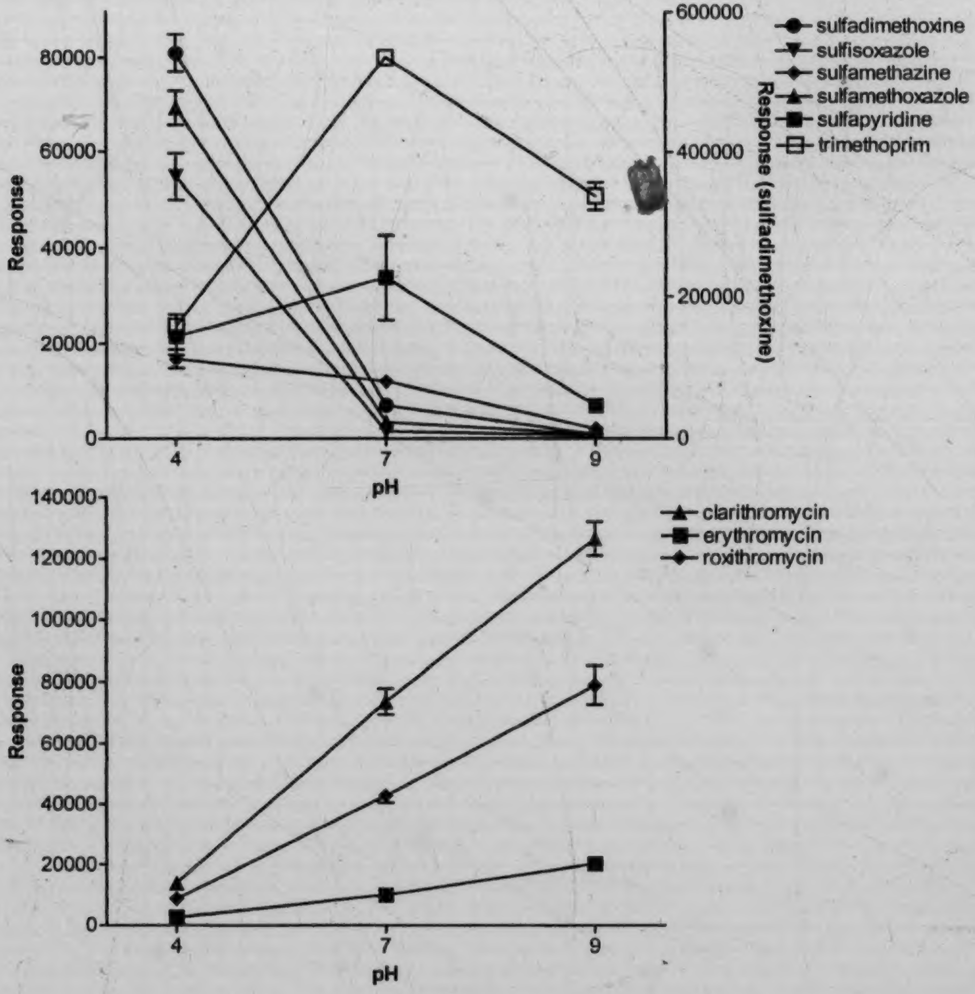


2.3.4 pH

The impact of solution pH on the extraction efficiency of the analytes was investigated using buffered analyte solutions prepared at pH 4, 7, and 9 (Figure 2.5). A wide range of pKa values are present within the studied group of compounds. Because the neutral form of an analyte is predominantly extracted by a non-ionic polymeric type fiber such as the CW/TPR (Aresta et al. 2003, Aresta et al. 2005, Chou and Lee 2005) pH was expected to affect the extraction efficiency of the various analytes in a compound-specific manner (Figure 2.1).

Most of the sulfonamides showed significantly greater extraction efficiency at a pH of 4; sulfapyridine and sulfamethazine also showed comparable extraction efficiency at pH 7 (Figure 2.5). When solution pH is increased, the acidic amide functionality (-NH) present in sulfonamides is deprotonated to be anionic. This moiety's pK_a is higher than that of the basic amine group (-NH_2), so at pH values above the amide pK_a, sulfonamide speciation favors an overall negative charge (Qiang and Adams 2004). This corresponds to a decrease in extraction efficiency. Under pH conditions between the amide and amine pK_a values (e.g., pH 4) which are between 1.32–2.13 and 5–6.08, respectively, (Qiang and Adams 2004, Wishart et al. 2006) (Figure 2.1), sulfonamides are predominantly in the neutral form with a corresponding increase in extraction efficiency as observed (Figure 2.5). The increased extraction efficiency of the sulfonamide antibiotics at a lower pH is consistent not only with the pK_a values of these compounds, but also with the results found by Balakrishnan et al. (2006) in their study of several sulfonamide antibiotics, in which an extraction pH of 4.5 was optimal. The sulfonamides which showed comparable extraction efficiency at pH 7 had higher amide pK_a values (7.49 and 8.43 for sulfamethazine and sulfapyridine, respectively) (Geiser et al. 2005, Qiang and Adams 2004, Wishart et al. 2006) than the other sulfonamides under consideration, and thus a larger fraction of these sulfonamide antibiotics were neutral at that pH. Extraction efficiency of trimethoprim was maximized at a pH of 7, while greatest extraction for the macrolides was observed at pH 9 (Figure 2.5). These results are consistent with the pK_a

Figure 2.5: Effect of solution pH on SPME extraction efficiency of sulfonamides and trimethoprim (top) and on macrolides (bottom)
Sulfadimethoxine response on right y-axis in top panel; all other analyte responses on left y-axis. Error bars represent standard error of the mean for each data point (n=6). Absence of error bars indicates that symbol is larger than error bars.



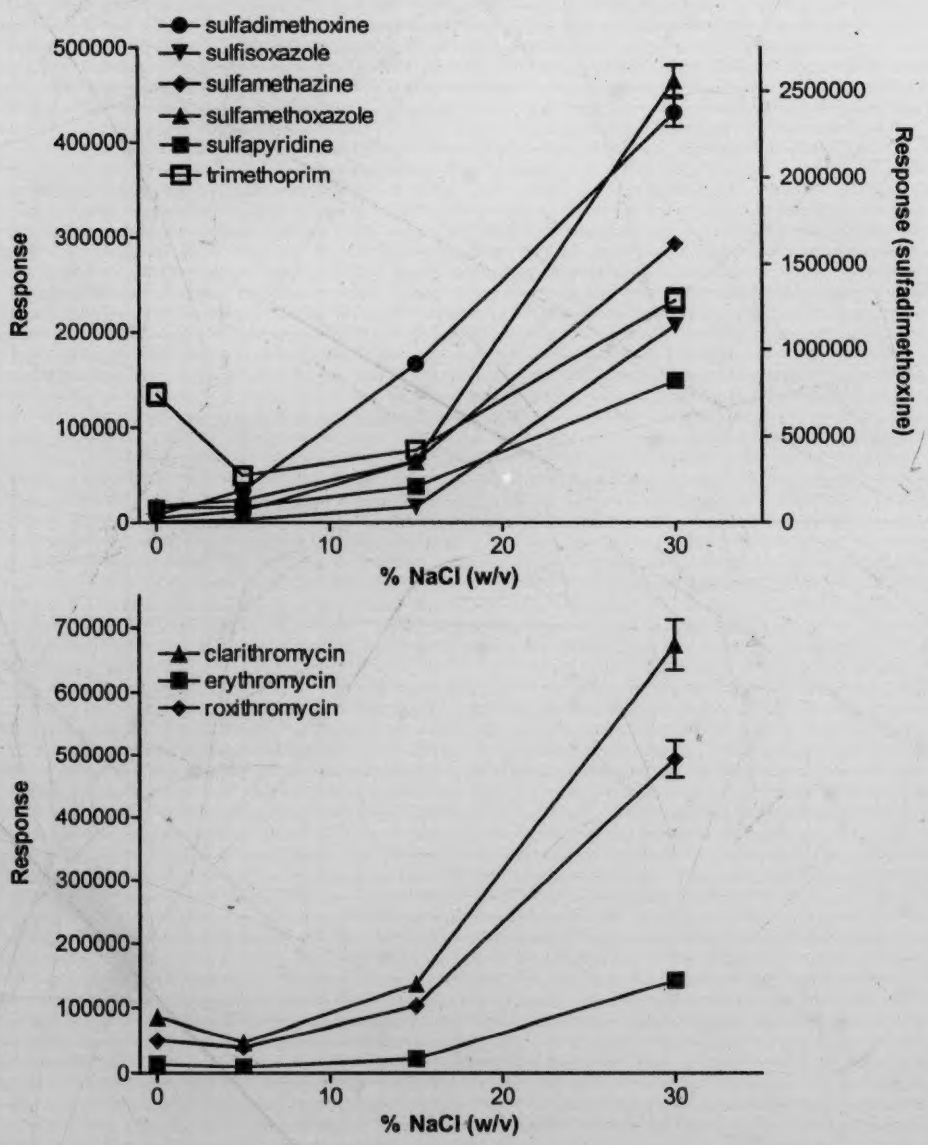
values associated with the compounds (Figure 2.1). Trimethoprim has pK_a values of 3.23 and 6.76 (Figure 2.1) in its two basic amine groups (Qiang and Adams 2004), so both are deprotonated under basic conditions as observed (Figure 2.5). The macrolides, which have basic dimethylamine groups [-N(CH_3)$_2$] that can gain a proton at pH values below their respective pK_a values of 8.74 to 9.17 (Figure 2.1) (Qiang and Adams 2004, Wishart et al. 2006), are thus more likely to be in their neutral forms at higher pH values (Figure 2.5).

Numerous other studies have optimized SPME pH for a single class of compounds or for individual compounds. In many of these studies, the pH chosen for extraction was optimal for all or most of the compounds. Balakrishnan et al. (2006) chose an extraction pH of 4.5 for the extraction of sulfonamide antibiotics in wastewater as greatest extraction efficiency was achieved for nearly all of the compounds at that pH. Rodriguez et al. (2004) found that a pH of 3 was optimal for all 5 NSAID compounds investigated in their work on optimizing a SPME method for these compounds. Given our goal of developing SPME extraction conditions for quantifying across multiple of antibiotics more likely to be found in environmental waters, we chose to consider further extraction at pH 4 and 7 for extraction of wastewaters, despite the greater macrolide extraction efficiency at basic pH.

2.3.5 *Ionic strength*

The effect of increasing solution ionic strength was investigated for SPME method optimization. Previous studies have shown that the addition of a neutral salt, such as sodium chloride, can significantly increase the extraction efficiency of polar organic compounds by SPME (Aresta et al. 2005, Balakrishnan et al. 2006, Lock et al. 1999, Lord and Pawliszyn 1997). The increased extraction of compounds from aqueous solution in the presence of salt can be explained by the "salting out" phenomenon. The addition of a neutral salt to the sample solution can result in a shift in equilibrium to favor the sorption of analyte to the fiber coating. To evaluate the effect of solution ionic

Figure 2.6: Effect of NaCl addition on SPME extraction efficiency of sulfonamides and trimethoprim (top) and on macrolides (bottom)
Sulfadimethoxine response on right y-axis in top panel; all other analyte responses on left y-axis. Error bars represent standard error of the mean for each data point (n=6). Absence of error bars indicates that symbol is larger than error bars.



strength, extractions of 100 μg/L analyte solutions at ionic strengths varying from 0 to 30% (w/v) sodium chloride were performed. The addition of sodium chloride increased significantly the extraction of all compounds, compared to extractions performed with no added salt (Figure 2.6). This is in agreement with previous studies.

Based on the observed trend of increasing extraction efficiency with increasing ionic strength, a concentration of 30% (w/v) NaCl was chosen for further experiments. NaCl concentrations above 30% (w/v) were not considered in this study, as amounts beyond 30% (w/v) approached solution saturation. To ensure consistent solution composition, and to avoid excess salt in the extraction solution, 30% (w/v) was the upper limit considered for salt content.

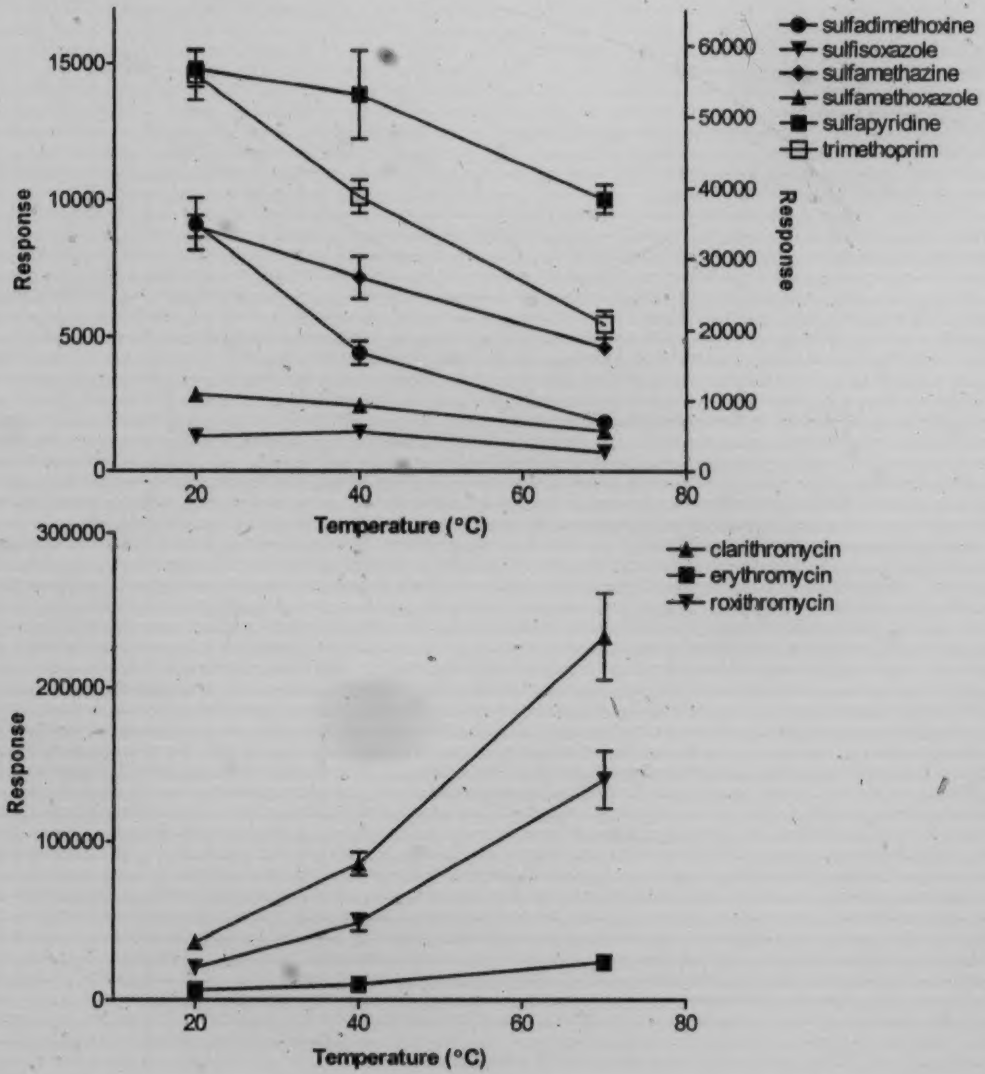
2.3.6 Temperature

An additional consideration in optimizing the SPME method was the temperature at which the extractions are done. Changes in extraction efficiency may be observed with an increase in temperature, due to two distinct and competing processes: increased analyte diffusion leading to an increase in extraction mass, and decreased analyte partition coefficients between the fiber and sample matrix leading to decreased extraction mass (Fernandes et al. 2007, Lord and Pawliszyn 2000b). Extractions of 100 µg/L analyte solutions (pH 7, 30% (w/v) NaCl) were carried out at temperatures of 20°C, 40°C and 70°C. The mass of analyte extracted decreased with increasing temperatures for all compounds except for the macrolides (Figure 2.7). An increase in extraction mass with increasing temperatures was also observed in the solid phase microextraction of several tetracycline antibiotics (tetracycline, oxytetracycline, minocycline, demeclocycline, anhydrotetracycline, 4-epianhydrotetracycline, methacycline) from milk (Lock et al. 1999), and several selective serotonin reuptake inhibitors (venlafaxine, fluvoxamine, fluoxetine, citalopram, sertraline) from environmental waters (Lamas et al. 2004). Others have reported a decrease in extraction mass with increasing temperature. Wu and Huang (1999) reported a decrease in extraction efficiency by the CW/TPR fiber for the extraction of several aromatic amines (benzidine, 3,3'-dimethylbenzidine, 4-aminobiphenyl, 3,3'-dichlorobenzidine, 2- naphthylamine, 4-aminoazobenzene) at elevated temperatures, and Aresta et al. (2005) reported similar results from their study of SPME of naproxen from urine. In cases where increases in temperature yield decreased extraction efficiency, the decreased analyte partition coefficients between fiber and sample may have been the dominating factor. In the present study, the sulfonamides and trimethoprim adhered to this behavior (Figure 2.7).

In the case of the macrolides, the dominating factor may be the increased diffusion of analyte to the fiber, resulting in increased extraction amount with higher temperatures. The larger size of the macrolide compounds relative to the other compounds may support why increased diffusion is the dominating process for the former, but not for the latter.

Despite the increasing extraction noted for the macrolides at higher temperatures, room temperature (20°C) was chosen for wastewater extractions. This was done to simplify the extraction procedure, and accommodate the most compounds within one set of extraction conditions, as previously stated.

Figure 2.7: Effect of solution temperature on SPME extraction efficiency of sulfonamides and trimethoprim (top) and macrolides (bottom)
Sulfadimethoxine and trimethoprim responses on right y-axis in left panel; all other analyte responses on left y-axis. Error bars represent standard error of the mean for each data point (n=6).



2.3.7 Summary of optimal SPME conditions

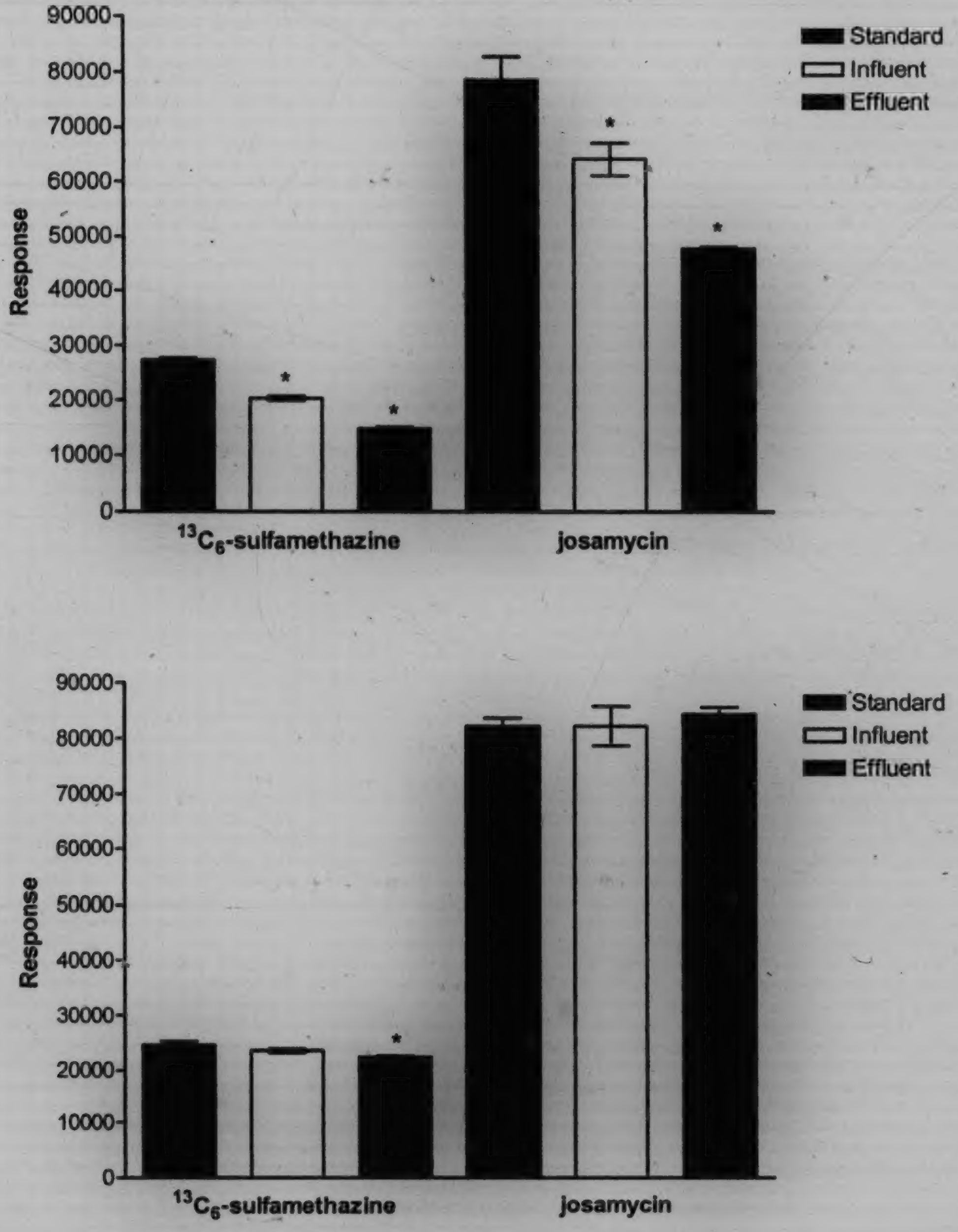
Overall, the extraction of the target analytes by SPME was significantly affected by all of the parameters investigated. As the goal of this study was to determine simultaneously antibiotics belonging to several classes, our optimization procedure minimized the overall number of different extraction procedures needed. As a result, simple and effective extraction procedures were established. The extraction conditions chosen for application to wastewater samples were: pHs of 4 and 7, 30% (w/v) NaCl, and extraction and desorption times of 30 minutes and 10 minutes, respectively. It is important to note that the results of the optimization experiments also provide information that would allow optimal extraction conditions to be chosen for a method targeting a specific antibiotic class or even a single compound, if so desired.

2.4 Matrix effects

Matrix effects in the analysis of water extracts generated by SPE have been widely reported (Benijts et al. 2004, Lindberg et al. 2004, Petrovic et al. 2005, Richardson and Ternes 2005). The electrospray source is very susceptible to the plethora of co-extracted matrix components in SPE wastewater extracts, and as a consequence, significant signal suppression or enhancement of target analytes is common (Gros et al. 2006). SPE extractions using the method of standard additions can be used to compensate for these matrix effects, but the disadvantages of this procedure are twofold: first, matrix effects are not eliminated, they are merely compensated for and second, performing standard additions with SPE is very time-consuming and not highly practical. To evaluate and compare the matrix effects in analysis that result from wastewater extracts generated by SPE and SPME at pH 7, the two internal standards were added to all standards and samples. The internal standards, $^{13}C_6$-sulfamethazine and josamycin are structurally similar to the target analytes; therefore the internal standards were used as model compounds for investigation of matrix effects, since they were not already present in wastewater. Matrix effects observed for the internal standards are an approximation of those expected for the target analytes. Suppression of signal was apparent for both internal standards in SPE wastewater extracts compared to standards made in nanopure water (Figure 2.8). These differences were significant (ANOVA, Tukey-Kramer post-hoc test, $P<0.05$ for all statistical tests in this section) for both internal standard responses in both influent and effluent compared to the nanopure standards. The greatest degree of suppression for the internal standards was observed in effluent, which may appear counterintuitive as influent is a more complex matrix. However, in the SPE procedure, five times more effluent (500 mL) than influent (100 mL) was extracted, so overall more matrix material was collected on the solid phase during the extraction of effluent samples.

Clearly, the coextraction of matrix by the SPE process can lead to significant ion suppression of analyte molecules in analysis, as previously observed for antibiotic quantification in wastewaters by LC-ESI-MS/MS (Benijts et al. 2004, Lindberg et al. 2004, Nikolai et al. 2006). In contrast, the SPME wastewater extracts showed no significant difference in signal for either internal standard compared to nanopure water standards (Figure 2.8). Although a statistical difference was found between the nanopure standard and effluent extracts for the $^{13}C_6$-sulfamethazine internal standard, that result is likely due to the high degree of precision associated with these measurements (Figure 2.8), and is not necessarily an indication of matrix effects. The response of the internal standards in the influent and effluent SPME extracts compared to the nanopure standard

Figure 2.8: Response of internal standards $^{13}C_6$-sulfamethazine and josamycin
*Response in SPE (top) and SPME (bottom) wastewater extracts and standards. n = 6 for SPE; n = 7 for SPME. Error bars represent standard error of the mean. * indicates statistical difference from standard (ANOVA, Tukey-Kramer post-test, p<0.05).*



indicates the absence of significant matrix effects in the SPME standards. This is evidence that very little of the sample matrix was transferred to the final extract, unlike the case for SPE extracts, where significant transfer of sample matrix to final extracts occurred. Visual comparison of the SPE and SPME extracts further confirmed that transfer of sample matrix was far more significant for the SPE extracts, as they were distinctly colored by matrix material, while the SPME extracts were not. The absence of matrix interferences in SPME final extracts was also observed for SPME of selective serotonin reuptake inhibitors from influent spiked with urine (Lamas et al. 2004), as well as SPME of parabens from several water matrices (Canosa et al. 2006). Our results, and those of previous studies, suggest that SPME is an alternative to SPE in applications where matrix effects are expected to be significant.

2.5 Method performance

To evaluate the application of the optimized SPME method, extractions were performed on wastewater. Seven-point standard addition curves at spike levels ranging from 0 to 5 µg/L were prepared in influent and effluent at pHs of 7 and 4 with 30% NaCl (w/v) added in each case. For comparison, extractions of the influent and effluent water were also done with the SPE method described previously. Calibration curves for the SPME and SPE methods can be found in Appendix C. While the SPME method performed well at a pH of 7, extractions done at a pH of 4 yielded poor results, which had also been observed in previous experiments we conducted. Sorption of target analytes to the SPME fiber was decreased and/or inconsistent in wastewater acidified to pH 4. Under these pH conditions, the turbidity of the wastewater solution was greatly diminished in comparison to wastewater at ambient pH, suggesting an increased solubility of matrix components at a lower pH. Increased coextraction of organic material by SPE sorbents has been noted at lower pH values (Weigel et al. 2004) (e.g., protonation of carboxylic moieties in organic material, rendering it neutral and amenable to sorption by extraction phases), and may explain the poor results achieved using SPME at a lower pH. An increase in dissolved organic matter in the wastewater resulting from a decrease in pH may interfere with the sorption of the target analytes by the fiber, thus limiting the usefulness of a lower pH for SPME extractions for this application.

The performance of the SPME method (pH 7) was compared to that of the SPE method (Table 2.2) through calculation of limits of detection (LOD) and limits of quantification (LOQ) for both methods, as well as determination of the correlation coefficients of the SPME standard addition curves. The SPME standard addition calibration curves were fit to linear equations with resulting r^2 values between 0.9080 to 0.9992 (Table 2.2), indicating a strong correlation for all analytes. The sulfonamide antibiotic sulfisoxazole was not detectable by the SPME method, so no values are shown for this analyte. In optimization experiments sulfisoxazole displayed a relatively weak affinity for the CW/TPR fiber. It is therefore not surprising that, at low concentrations, and in a wastewater matrix, it was not successfully extracted. The LOD and LOQ values for SPME were calculated for both influent and effluent as the mass of analyte that produced a signal-to-noise ratio of 3 and 10 for LOD and LOQ, respectively, with the resulting value being divided by the water sample size. The same procedure was used to calculate LOD and LOQ values for SPE in influent and effluent. The internal standard calibration curves used for the SPE method were prepared from standards in methanol with analyte concentrations ranging from 1 to 100 μg/L and internal standards present in all standards at 50 µg/L. The r^2 values were typically 0.99 or greater, indicating a strong correlation.

Table 2.2: Linearity, limits of detection (LOD), and limits of quantification (LOQ) for SPE and SPME methods in wastewater influent and effluent

	Solid phase extraction			Solid phase microextraction		
Compound	r^2	**LOD/LOQ influent (ng/L)**	**LOD/LOQ effluent (ng/L)**	r^2 **influent/ effluent**	**LOD/LOQ influent (ng/L)**	**LOD/LOQ effluent (ng/L)**
Sulfonamides						
Sulfapyridine	0.9930	3.8/13	1.4/4.8	0.9770/ 0.9952	61/200	59/200
Sulfamethoxazole	0.9865	4.5/15	1.8/6.1	0.9080/ 0.9799	410/1380	77/260
Sulfisoxazole	0.9932	6.1/20	1.5/4.9	n/a	n/a	n/a
Sulfamethazine	0.9960	1.6/5.2	0.94/3.1	0.9992/ 0.9989	61/200	38/130
Sulfadimethoxine	0.9917	0.83/2.8	0.42/1.4	0.9985/ 0.9961	27/91	13/43
Macrolides						
Clarithromycin	0.9898	2.6/8.7	1.3/4.3	0.9936/ 0.9336	4.8/16	11/35
Erythromycin	0.9939	2.0/6.8	1.7/5.8	0.9987/ 0.9987	12/39	20/66
Roxithr[illegible]	0.9966	0.41/1.4	0.08/0.27	0.9940/ 0.9565	2.8/9.2	4.1/14
Other						
Trimethoprim	0.9952	1.4/4.7	0.26/0.86	0.9962/ 0.9947	21/68	15/49

The LOD and LOQ values in influent and effluent derived for SPME were generally one to two orders of magnitude higher than those resulting from the SPE method. This result is not unexpected because SPE is an exhaustive extraction technique that collects analytes with affinity for the stationary phase until it is saturated, while SPME is an equilibrium technique in which only a portion of the analyte present sorbs to the fiber.

2.6 Concentrations of antibiotics in wastewaters

Of the nine target compounds, sulfamethoxazole, trimethoprim, erythromycin and clarithromycin and were measurable by both SPE and SPME (pH 7) in influent, effluent or both (Table 2.3). Sulfapyridine was also measurable by SPE but was below the LOQ for the SPME method. MRM chromatograms for analytes detected in the wastewater are shown in Appendix B. Taking into account the overall recovery of each compound by the SPE method, and the error associated with each measurement, the concentrations generated by the two methods are in reasonable agreement with one another (Table 2.3). For example, in both influent and effluent, the SPE concentrations for trimethoprim (0.26 and 0.27 µg/L for influent and effluent, respectively) were approximately half the SPME concentrations (0.46 and 0.44 µg/L for influent and effluent, respectively). However, the SPE method recovered only about half of the trimethoprim present in wastewater (Table 2.3). Taking the SPE recoveries into account, there was good agreement between the two sets of measurements. The concentrations determined by both methods for clarithromycin were also in good agreement (Table 2.3). The recovery of clarithromycin in wastewater by SPE was high (88% and 79% for influent and effluent, respectively). Therefore a direct comparison of the concentrations obtained by the two extraction methods is reasonable. The clarithromycin SPE concentrations were 1.39 and 0.91 µg/L in influent and effluent, respectively, while the corresponding SPME concentrations were 1.57 and 1.24 µg/L. Using standard additions to measure the SPME concentrations is advantageous because this method, unlike SPE, accounts for analyte recovery and eliminates the need for a recovery correction. In addition, isotopically labeled internal standards are not available for emerging pollutants such as antibiotics, and are expensive in any event. Standard addition allows for reliable quantification of all analytes (Miao et al. 2004), while the small amounts of sample used (1.5 mL) in our SPME method allows for straightforward preparation of standard addition calibration curves, which is often cumbersome to perform by SPE.

Table 2.3: Comparison of analyte concentrations in Edmonton Gold Bar wastewater.
Comparison of concentrations in influent and effluent collected on May 14, 2007 and measured by SPE and SPME. SPE measurements were made in triplicate and quantified by internal standard calibration. Single SPME measurements were made on influent and effluent wastewater samples by standard addition calibration. The %RSDs on SPME concentrations were calculated based on propagation of error of the slopes and intercepts of the standard addition calibration equations.

	INFLUENT			**EFFLUENT**		
Analyte	**SPE (µg/L) (%RSD)**	**% SPE recovery**	**SPME (µg/L) (%RSD)**	**SPE (µg/L) (%RSD)**	**% SPE recovery**	**SPME (µg/L) (%RSD)**
Sulfamethoxazole	0.65 (5)	66	1.37 (31)	0.29 (1)	87	nd
Trimethoprim	0.27 (7)	52	0.46 (16)	0.26 (3)	51	0.44 (20)
Erythromycin	0.044 (19)	88	0.086 (50)	0.092 (5)	61	0.16 (26)
Clarithromycin	1.39 (2)	88	1.57 (7)	0.91 (1)	79	1.24 (28)

It is clear from the measurements that SPE was the more precise of the two methods with percent relative standard deviations (%RSD) between 1–19%, compared to SPME %RSDs between 7–50%. However, it is worth noting that with the exception of erythromycin in influent, %RSDs for the SPME method were no greater than 31%. The higher precision measurements obtained by SPE over SPME are not surprising given that SPE extracts, which result from exhaustive extraction of sample, contain a much higher concentration of the analyte than do the SPME extracts. Only a small portion of the low level of analyte present in wastewater will sorb to the SPME fiber coating; measurements of concentrations approaching the LOQs for this method are not surprisingly less precise.

For example, the concentration of sulfamethoxazole measured in influent is very close to the LOQ for the method, increasing the likelihood of error in measurement. Balakrishnan et al. (2006) also observed higher precision for SPE measurements compared to measurements obtained by SPME for the determination of recovery of sulfonamide antibiotics from wastewaters.

Despite the higher LOQs for the SPME method, most of the compounds present in wastewater were at concentrations above their respective LOQs (Table 2.2 and Table 2.3). The antibiotic concentrations reported here are similar to those found in other studies investigating concentrations of antibiotics in wastewaters. Miao et al. (2004) found maximum concentrations of 0.536 μg/L for clarithromycin, 0.838 μg/L for erythromycin and 0.871 μg/L for sulfamethoxazole in final effluents of eight WWTPs in five Canadian cities. Effluent wastewater samples collected from Georgia, California, and Arizona had sulfamethoxazole and trimethoprim at concentrations ranging from 0.395–0.575 μg/L and 0.040–0.705 μg/L, for the respective compounds (Renew and Huang 2004). Based on the concentrations we observed, and the similar concentrations reported elsewhere, we conclude that the higher SPME quantitation limits do not prohibit measurement of these analytes in typical wastewaters. Application of SPME to surface waters for these antibiotics is probably not feasible, as surface water concentrations are generally much lower than those found in wastewaters. Hao et al. (2006) found mean concentrations ranging from 0.20–2.0 ng/L, 0.10–6.9 ng/L and 0.50–38 ng/L for trimethoprim, erythromycin, and sulfamethazine, respectively, in Ontario, Canada surface waters receiving agricultural or urban inputs. Even at the upper limits of these concentration ranges, the SPME LOQs reported in the present study are insufficient to provide quantifiable measurements of the analytes under consideration (Table 2.2). With the exception of erythromycin, the concentrations in surface waters reported by Vanderford et al. (2003) for several of the target analytes in various Las Vegas surface waters were below the LOQs for our SPME method. Erythromycin (measured as erythromycin-H_2O) was present at 98 ng/L in the Las Vegas Wash, a body of water composed of ~90% treated wastewater effluent. Based on our calculated LOQ values for erythromycin in influent and effluent, it is likely that the SPME method could detect erythromycin at this concentration in surface waters. The SPME method may therefore be useful for the measurement of some of the target analytes in surface waters highly impacted by wastewater effluent, but is likely not useful for surface waters receiving less extreme urban or agricultural inputs. To extend the use of SPME to surface waters, a pre-concentration step, such as lyophilization, may be necessary.

2.7 Conclusions

Overall it appears the SPME method is a useful alternative to SPE in the determination of certain antibiotics from wastewater. The major limitations of the method are its higher limits of quantification, and secondarily, its lower precision measurements. Some advantages of the SPME method over typical SPE methods include: decreased sample volume requirements (100 mL vs. 4L), decreased cost, and ease of sample extraction. Sample preparation, extraction and subsequent processing steps using the SPME method require less time and labor than the SPE method. The most significant advantage of the SPME method over SPE methods is the elimination of matrix effects in analysis. Despite the attractive qualities of SPME, its high limits of quantification indicate that SPE is a more widely applicable and useful technique for trace analysis of antibiotics. However, our results indicate that SPME can be used to successfully measure some antibiotics in wastewater, and its use could likely be extended to include other antibiotics and pharmaceuticals. In some cases it may even be useful for measuring some analytes in surface waters.

2.8 Future Directions

The utility of the SPME method presented previously could be improved and expanded. The main limitation of this method is its higher limits of quantification, prohibiting the quantification of analytes occurring at levels below the method LOQs. This is especially true for aqueous matrices such as surface and ground water, where increasing dilution occurs with the distance from the source of contamination. Certain compounds may still be detectable in some surface waters, but the use of this SPME method is likely fairly limited for application to surface waters. To overcome this limitation would require the use of a pre-concentration step, such as lyophilization. The disadvantages of this approach, of course, are the additional time added to the overall sample processing, and the availability of the proper instrumentation.

Another issue observed with the SPME method was the inability to perform wastewater extractions under acidic conditions successfully. In optimization experiments, several of the investigated compounds demonstrated greater extraction efficiency under acidic conditions. If wastewater extractions can not be determined under the conditions determined to be optimal for a given compound or group of compounds, the use of the SPME technique is potentially compromised. In this situation, a clean-up step either before or after acidification of the sample may be useful. For non-charged, medium polarity compounds, adsorptive materials, such as silica gel and aluminum oxides can be used to remove polar matrix material. Gel permeation chromatography is useful for the separation of organic analytes from matrix components such as proteins, lipid and humic material (McArdell et al. 2006). The very small volume of water needed for SPME extractions means that clean-up steps would not be extremely lengthy. Sample clean-up steps have been frequently used prior to SPE in order to eliminate organic material from the wastewater; similar procedures could be used for clean-up prior to SPME.

The extension of this SPME method to include other antibiotics would be a worthwhile pursuit. Another important class of antibiotics, the fluoroquinolones, were investigated in the early stages of SPME method development. However, the behavior observed for these compounds during mass spectrometric analysis prohibited their inclusion in further SPME method development efforts. The response of the investigated fluoroquinolone antibiotics (ciprofloxacin, norfloxacin, enrofloxacin and internal standard lomefloxacin) was extremely poor by LC-MS/MS analysis, and was not able to be improved. This particular issue has not been noted in the literature, thus further development will likely yield good results.

SPME extraction of a wide range of pharmaceuticals has been demonstrated in a variety of biological matrices as well as environmental matrices. The potential for SPME to be successfully applied to the extraction of other pharmaceuticals from wastewaters is high, and further investigation is certainly warranted.

3 Appendices

Appendix A: Instructions for SPME and SPE of environmental water samples for determination of antibiotics

1. Materials and reagents needed

- Common supplies
    - Filtration apparatus
    - Glass fiber filters 1.6, 0.7 µm (precombusted at 450°C for 4 hours)
    - Nitrogen evaporator (e.g., N-EVAP from Organomation Inc.)
    - Analyte standards in methanol
    - Internal standards in methanol

- SPME materials and reagents
    - SPME fiber(s) (Supelco, Oakville, Canada)
    - SPME fiber holder(s) (Supelco, Oakville, Canada)
    - Ring stands and clamps
    - Magnetic stirrers
    - Micro stirbars (2 × 7 mm) (Fisher Scientific)
    - Volumetric flasks
    - Small sample vials (HPLC autosampler vials work well)
    - Sodium chloride (NaCl)
    - Buffering reagents (e.g., ammonium acetate/acetic acid for pH 4)
    - Desorption solvent (methanol or acetonitrile)

- SPE materials and reagents
    - SPE vacuum manifold (with large volume vacuum trap attached to collect water)
    - SPE PTFE sippers (e.g., from Scientific Products & Equipment, North York, Canada)
    - SPE cartridges (e.g., Oasis HLB 3 mL, 60 mg cartridges from Waters, Mississauga, Canada, or similar)
    - Erlenmeyer flasks (appropriate size for water samples)
    - Graduated cylinder (large, at least 500 mL capacity)
    - Parafilm
    - Test tubes
    - Drying tubes (make using a short Pasteur pipet; add small amount of glass wool, followed by ~5 cm column of sodium sulfate)
    - Small glass vials with PTFE caps (~10 mL capacity)
    - Syringe filters (e.g. PTFE, 0.22 µm – ensure compatibility with solvent)
    - Autosampler vials
    - Methanol

  - Nanopure water or equivalent (e.g., nanopure water from a Nanopure Ultrapure Water System (Barnsteam/Thermolyne, Dubuque, IA, USA))

2. *Sample processing:* ***SPME*** (process wastewater in Biosafety cabinet and follow all biosafety procedures for safe handling of wastewater)
   - 2.1. Filter required volume of water through 1.6 and 0.7 µm glass fiber filters (precombusted for 4 hours at 450°C). Filter enough water to do all extractions (1.5 mL is required for each sample) plus some extra water in case of spillage.
   - 2.2. Adjust ionic strength by adding water to a pre-measured amount of salt (NaCl) in the appropriate sized volumetric flask (e.g., for 100 mL of 30% (w/v) NaCl water, add 30 grams of NaCl to a 100 mL volumetric flask and fill to the mark with water).
   - 2.3. Adjust solution pH using desired buffers (e.g., acetate/acetic acid for acidic pH, phosphate buffers for neutral pH). This should be done after ionic strength adjustment to ensure that the pH effects of salt are accounted for. A pH meter can be set up near the Biosafety cabinet using a cart or other suitable surface.
   - 2.4. To prepare standards for a standard addition curve, add appropriate amounts of analyte stock solution (in methanol) to small volumetric flasks. Using the nitrogen evaporator, evaporate methanol to dryness. The presence of methanol in the samples will have a negative effect on extraction, so ensure the methanol is completely evaporated. Bring the flask to volume with the water.
   - 2.5. Aliquot water samples (1.5 mL) into autosampler vials containing a micro stirbar (2 × 7 mm). **Make sure the stirbars have been thoroughly cleaned, otherwise sample contamination can occur. To clean stirbars, wash with water and regular lab detergent, rinse with distilled water, put in nanopure water and allow to stir on magnetic stir plate (~5 minutes), then put in methanol and allow to stir on magnetic stir plate (~5 minutes). At the end of the day, the stirbars can be put through the current regular wash cycle using Contrad cleaner. Put some of the Contrad solution from the large containers into a small beaker and allow the stir bars to soak.**

3. *Extraction procedure: **SPME*** (extract wastewater in Biosafety cabinet)

   3.1. Attach SPME fiber to fiber holder; this apparatus will be referred to as the "fiber assembly."

   3.2. Clamp the fiber assembly to a ring stand such that the fiber assembly can be lowered into, and raised out of, the sample.

   3.3. Set up a magnetic stirrer at the base of the ring stand, directly below the fiber assembly.

   3.4. Position a clamp above the magnetic stirrer, but below the fiber assembly, to hold the autosampler vial containing sample.

   3.5. Secure the uncapped sample in the clamp (see 3.4), ensuring that the opening to the sample is directly below the fiber. Make necessary adjustments to positioning to ensure that stirring is smooth and consistent.

   3.6. CAREFULLY lower the fiber assembly into the sample. Immerse the entire fiber while avoiding the stir bar.

   3.7. Start timing the extraction as soon as possible.

   3.8. After the timer has been started, make any necessary adjustments to the fiber position.

   3.9. When the extraction time is up, carefully raise the fiber assembly out of the sample. **Retracting the fiber after an extraction, especially when using high concentrations, is not recommended. Previous experience has shown that contamination of the fiber guide occurs when retracting the fiber into the fiber guide after extraction. It is difficult, if not impossible, to clean the fiber guide. The contamination can affect subsequent measurements, leading to false results. At the end of a series of extractions, the fiber may be retracted after thorough cleaning. Because the fiber is exposed much of the time, extreme care must be taken not to break it. The fibers are very fragile and expensive.**

4. *Desorption procedure:* ***SPME***

4.1. Desorption of analytes off the fiber is done in 1.5 mL of an appropriate solvent (acetonitrile, methanol) in an autosampler vial containing a micro stirbar.

4.2. Follow instructions as per extraction procedure (see 3.5 - 3.9).

4.3. After desorption, the fiber is clean and ready for the next extraction. If carry-over is suspected (i.e., some analyte remains on fiber after desorption) increase desorption time. Visually inspect the fiber after desorptions to make sure it is clean. If it appears dirty or coated, a cleaning step before the next extraction may be desirable. This can be done with a combination of water and methanol.

4.4. At the end of a series of extractions, a more thorough cleaning of the fiber with additional methanol and nanopure water should be done.

5. *Extract processing:* ***SPME***

5.1. The resulting extract (in desorption solvent) can be evaporated using a nitrogen evaporator. Place vial under a N-EVAP port. Direct tip of port to vial, then adjust nitrogen flow so that solvent shows a meniscus but does not bubble. Continue until solvent is evaporated, then cap vial until step 5.2 is performed.

5.2. To prepare the extract for analysis, reconstituting the extract in the HPLC mobile phase is recommended. This is particularly important when using large injection volumes (>20 μL) as the peak shape will be negatively impacted if a solvent stronger than the mobile phase is used. To add internal standard, make the desired concentration of internal standard in the mobile phase. This can then be used to reconstitute the extract.

5.3. For environmental samples, a volume of 75 μL is recommended for reconstitution of the extract. This volume allows for a large injection volume (50 μL) while avoiding unnecessarily diluting the extract.

5.4. After addition of mobile phase, shake the vials thoroughly. This can be done by hand. A vortexer is also useful for this purpose.

5.5. Transfer the contents of the vial to a 200 µL insert. Remove the stirbar from the vial, and place the insert in the vial. The extract is now ready for analysis, using the chromatographic and MS methods described in section 2.2.5 of this thesis. Keep in mind that the 75 µL extract will only be good for one injection.

6. *Sample processing:* ***SPE*** (process wastewater in Biosafety cabinet and follow all biosafety procedures for safe handling of wastewater)

6.1. Filter required volume of water (100 mL for influent samples, 500 mL for effluent samples) through 1.6 and 0.7 µm glass fiber filters (precombusted for 4 hours at 450°C).

6.2. Add required amount of water to Erlenmeyer flasks (100 mL/influent sample; 500 mL/effluent sample). To evaluate recovery of SPE procedure, recovery samples are used. Spike the appropriate amount of analyte standard into an Erlenmeyer flask prior to addition of water; a spike level of 100 ng should be sufficient. Ensure that the solvent (i.e., methanol) content of the sample is no greater than 0.05% of the total volume.

7. *Extraction procedure:* ***SPE***

7.1. Set-up vacuum manifold with vacuum trap. Arrange SPE cartridges on the manifold, making sure all ports are in the closed position.

7.2. Turn on vacuum and adjust until appropriate level of vacuum is achieved (about 0.2 bar of vacuum).

7.3. Condition cartridges by sequentially passing 2× cartridge volumes of methanol, followed by 2× cartridge volumes of nanopure water through each cartridge. Try to avoid letting cartridges go dry during conditioning procedure.

7.4. Fill cartridges to top with nanopure water. Fill sippers with nanopure water and secure to appropriate cartridge – the other end of the sipper needs to be in the appropriate sample.

7.5. Open the ports one-by-one to ensure that sample is being delivered through the sipper to the cartridge. Make any necessary adjustments to the vacuum.

7.6. Allow entire sample to pass through cartridge at an approximate flow rate of 2-3 mL/min, which is a steady drip. This can take time, so be patient. Samples that have been adjusted to higher pHs may need to be refiltered prior to SPE – this has been observed with wastewater at a pH of 9-10.

7.7. Allow cartridges to sit under vacuum for an additional 15 minutes after all sample has been passed through.

8. *Extract processing:* **SPE**

8.1. Parafilm SPE cartridges to handle them outside the Biosafety cabinet.

8.2. Elute analytes by placing each cartridge in a test tube and adding 2 × 3 mL of eluent (methanol) to the cartridge. Centrifuge for ~5 min for the first 3 mL volume and for ~10 min for the second 3 mL volume.

8.3. Pass the extract (6 mL) through a drying tube. Follow the extract with a small amount of methanol to ensure all analyte is removed from drying column.

8.4. Filter the extract using a syringe filter.

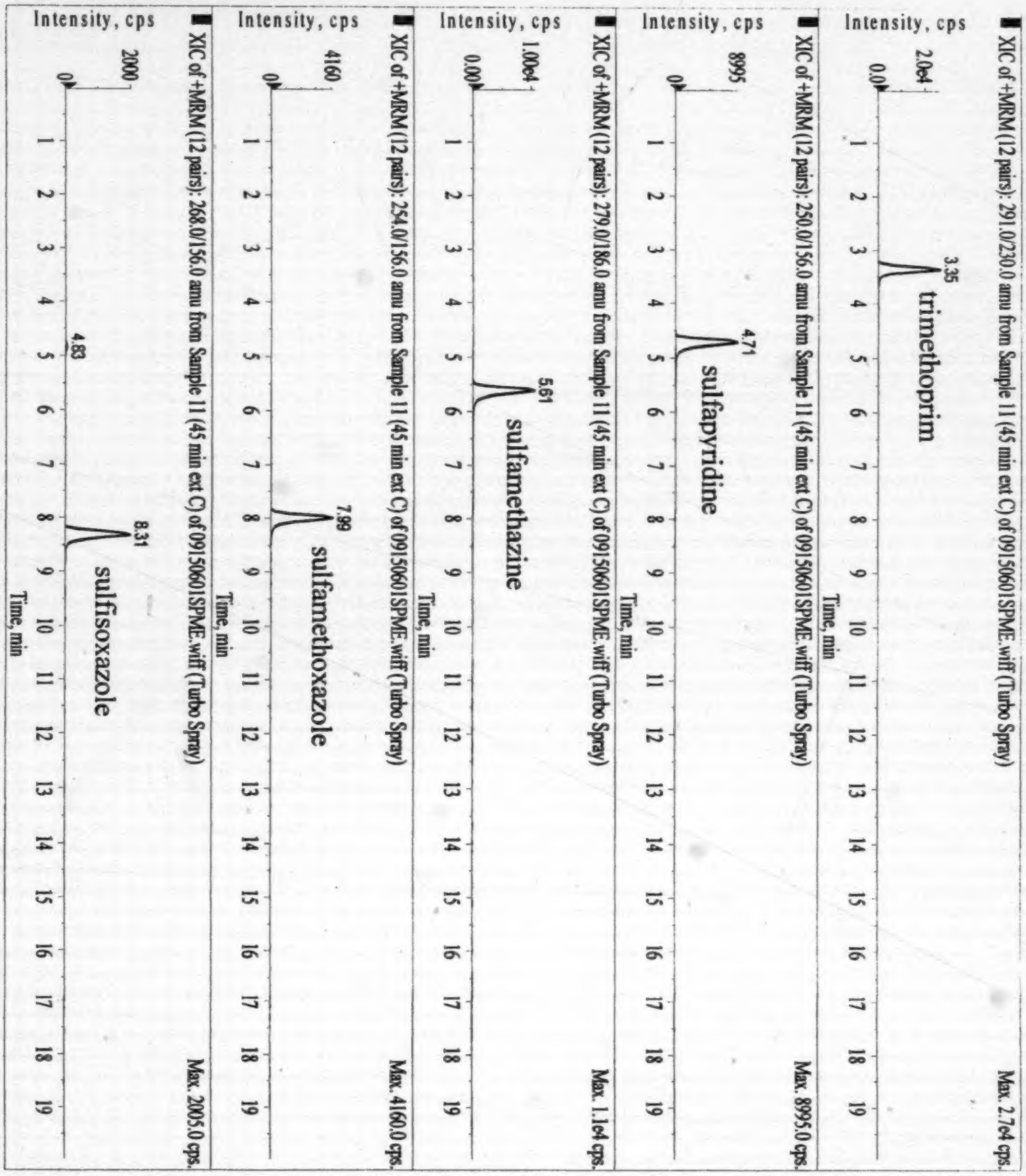
8.5. Evaporate extracts using autosampler vials (add extract gradually) and a nitrogen evaporator.

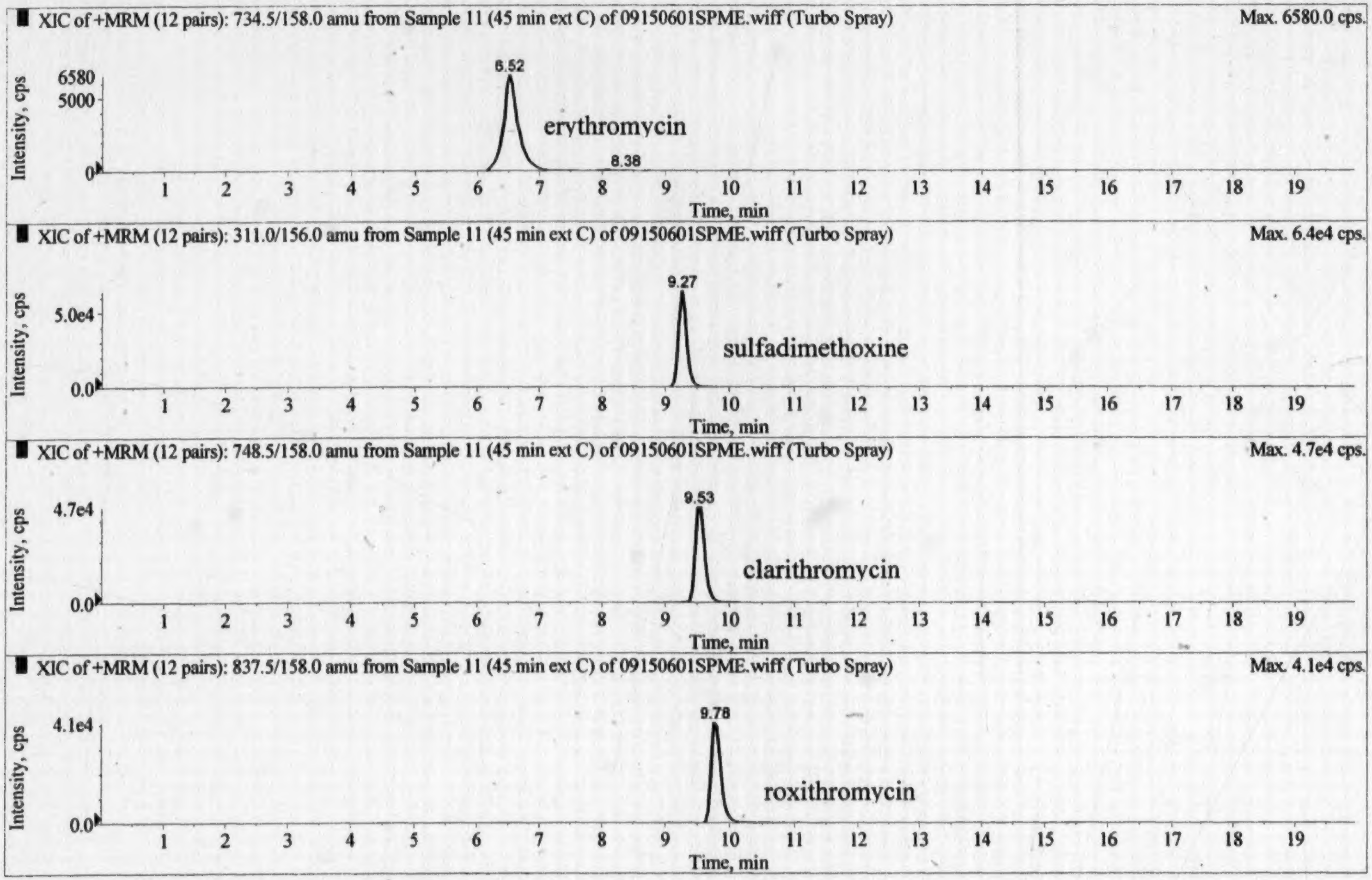
8.6. Reconstitute extract with internal standard solution and mobile phase (or other appropriate solvent). The extract is now ready for analysis, as described in section 2.2.5 of this thesis.

Appendix B: Representative MRM chromatograms of target compounds

MRM chromatograms of target analytes in: 1) clean standard 2) influent and effluent SPME extracts and 3) influent and effluent SPE extracts. For all chromatograms, chromatographic and mass spectrometric conditions are as stated in section 2.2.5 of this thesis.

1) MRM chromatogram of target analytes in SPME extract resulting from extraction of 100 ppb standard.




XIC of +MRM (12 pairs): 734.5/158.0 amu from Sample 11 (45 min ext C) of 09150601SPME.wiff (Turbo Spray)
Max. 6580.0 cps.
Intensity, cps
6580
5000
0
6.52
erythromycin
8.38
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
Time, min
XIC of +MRM (12 pairs): 311.0/156.0 amu from Sample 11 (45 min ext C) of 09150601SPME.wiff (Turbo Spray)
Max. 6.4e4 cps.
Intensity, cps
5.0e4
0.0
9.27
sulfadimethoxine
Time, min
XIC of +MRM (12 pairs): 748.5/158.0 amu from Sample 11 (45 min ext C) of 09150601SPME.wiff (Turbo Spray)
Max. 4.7e4 cps.
Intensity, cps
4.7e4
0.0
9.53
clarithromycin
Time, min
XIC of +MRM (12 pairs): 837.5/158.0 amu from Sample 11 (45 min ext C) of 09150601SPME.wiff (Turbo Spray)
Max. 4.1e4 cps.
Intensity, cps
4.1e4
0.0
9.78
roxithromycin
Time, min

2) SPME wastewater extraction: MRM chromatograms of target analytes present in May 14, 2007 unfortified wastewater influent (top) and effluent (bottom).

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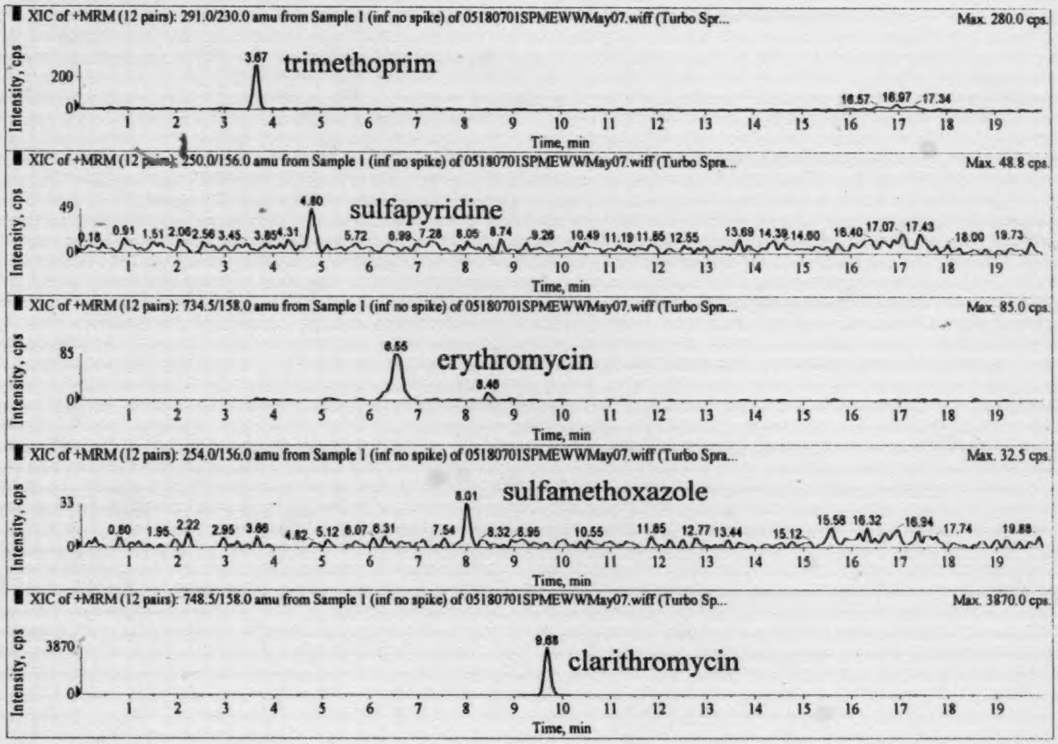

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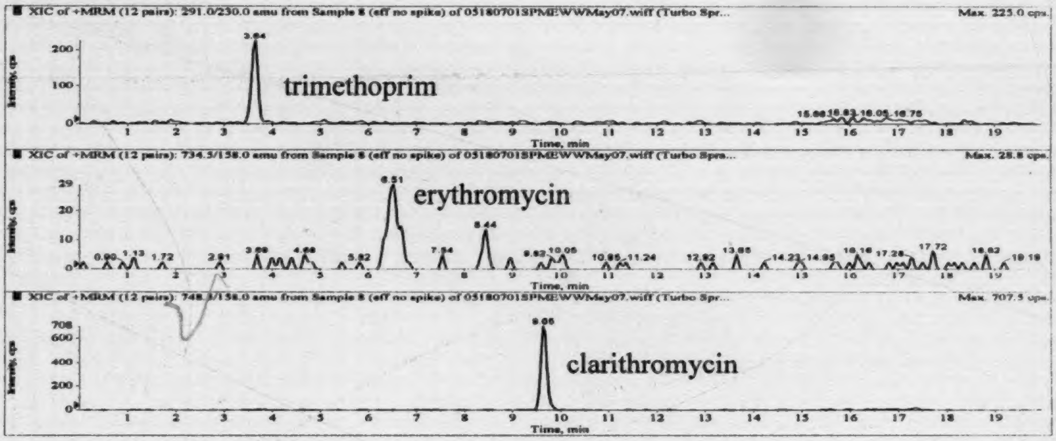

3) SPE wastewater extraction: MRM chromatograms of target analytes present in May 14, 2007 unfortified wastewater influent (top) and effluent (bottom).

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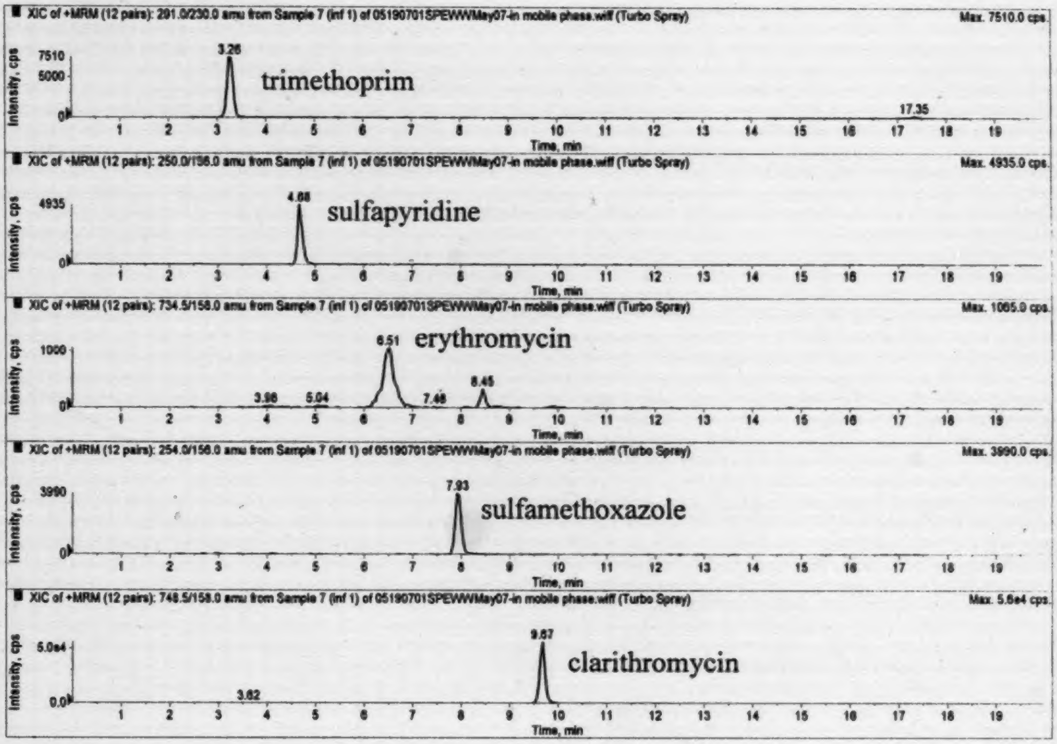

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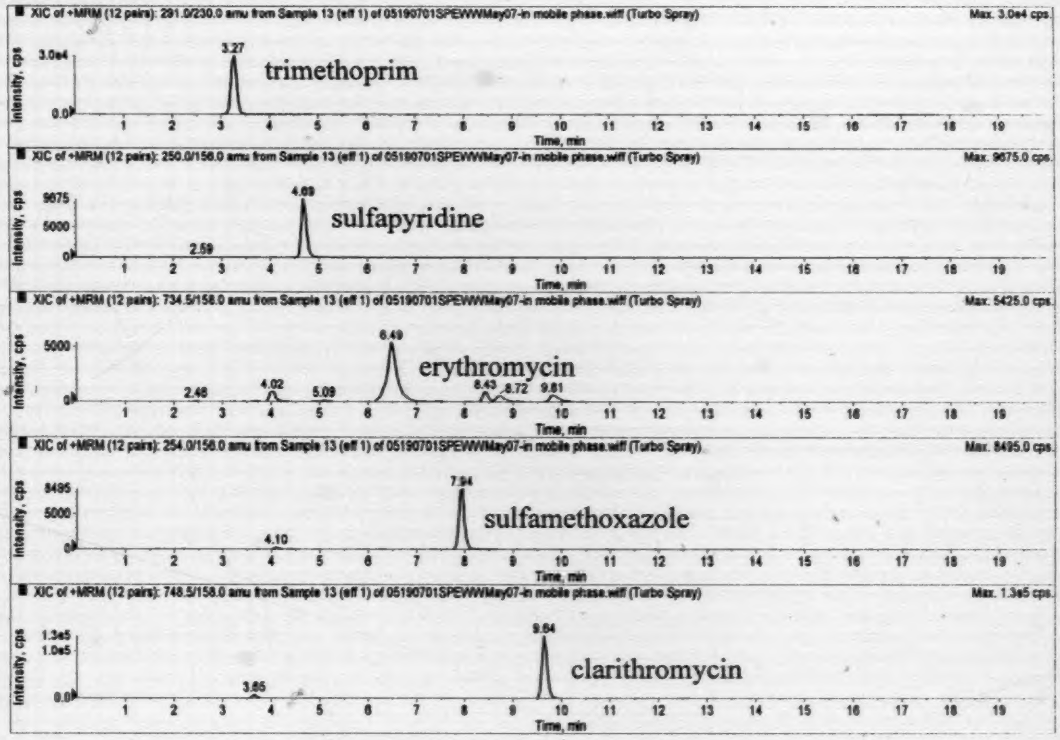
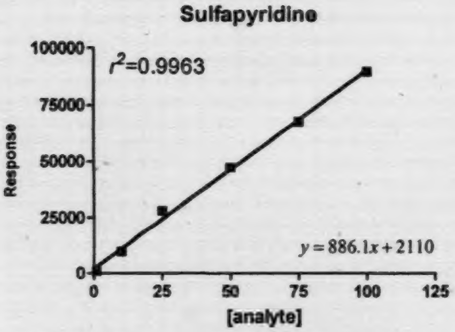
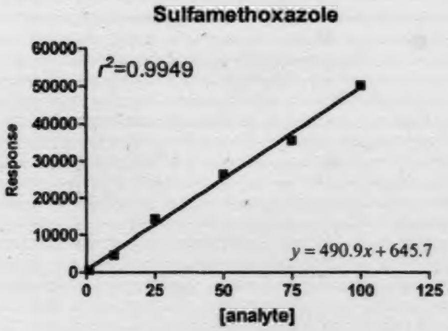
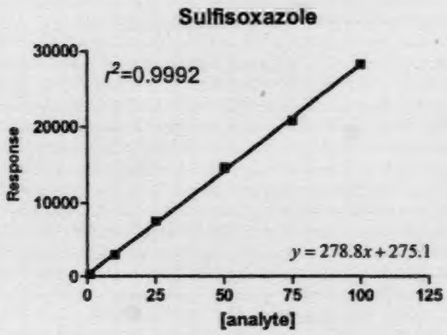

XIC of +MRM (12 pairs): 291.0/230.0 amu from Sample 13 (eff 1) of 05190701SPEWWMay07-in mobile phase.wiff (Turbo Spray)
Max. 3.0e4 cps.
Intensity, cps
3.0e4
0.0
3.27
trimethoprim
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Time, min
XIC of +MRM (12 pairs): 250.0/156.0 amu from Sample 13 (eff 1) of 05190701SPEWWMay07-in mobile phase.wiff (Turbo Spray)
Max. 9675.0 cps.
Intensity, cps
9675
5000
0
4.69
2.59
sulfapyridine
Time, min
XIC of +MRM (12 pairs): 734.5/158.0 amu from Sample 13 (eff 1) of 05190701SPEWWMay07-in mobile phase.wiff (Turbo Spray)
Max. 5425.0 cps.
Intensity, cps
5000
0
6.49
erythromycin
2.48
4.02
5.09
8.43
8.72
9.81
Time, min
XIC of +MRM (12 pairs): 254.0/156.0 amu from Sample 13 (eff 1) of 05190701SPEWWMay07-in mobile phase.wiff (Turbo Spray)
Max. 8495.0 cps.
Intensity, cps
8495
5000
0
7.94
sulfamethoxazole
4.10
Time, min
XIC of +MRM (12 pairs): 748.5/158.0 amu from Sample 13 (eff 1) of 05190701SPEWWMay07-in mobile phase.wiff (Turbo Spray)
Max. 1.3e5 cps.
Intensity, cps
1.3e5
1.0e5
0.0
9.64
clarithromycin
3.65
Time, min

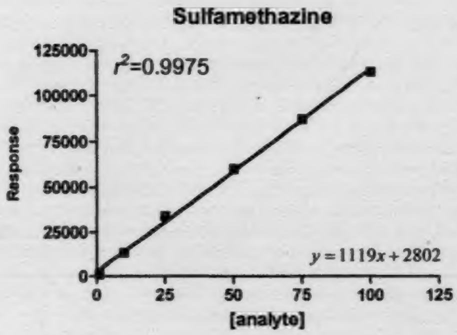
Appendix C: Calibration curves

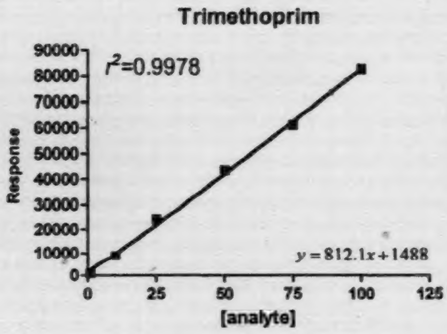
1) External calibration curves generated by injection of analyte standards (in methanol). Analyte concentrations are 1–100 µg/L. Line equation and r^2 values are displayed on plots. Concentration of analyte, [analyte], is in ng/mL.

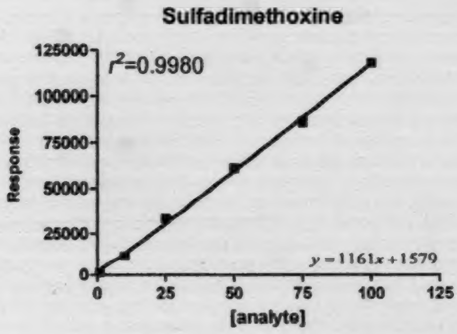


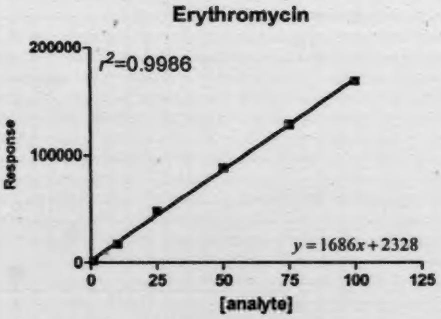


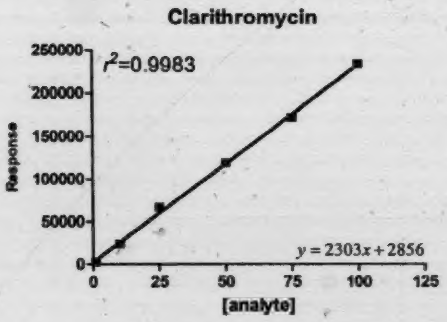


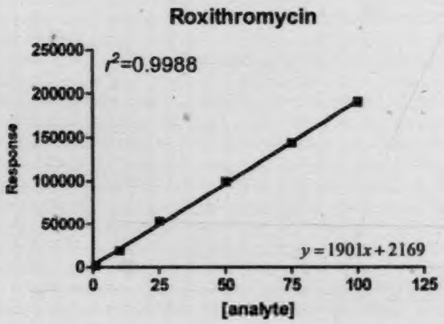




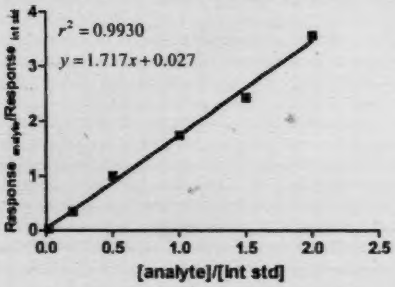


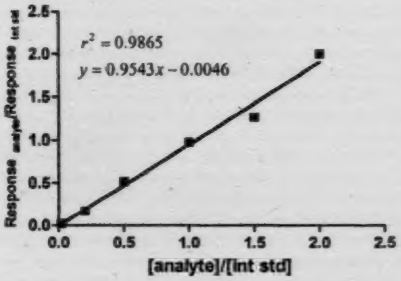

Erythromycin
r^2=0.9986
200000
100000
0
Response
$y = 1686x + 2328$
0
25
50
75
100
125
[analyte]

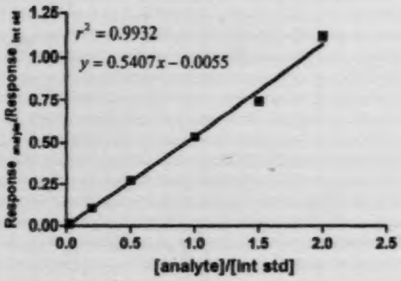

Clarithromycin
r^2=0.9983
250000
200000
150000
100000
50000
0
Response
$y = 2303x + 2856$
0
25
50
75
100
125
[analyte]

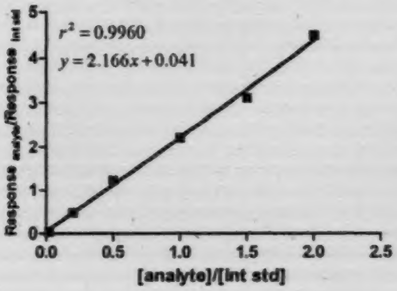

Roxithromycin
r^2=0.9988
250000
200000
150000
100000
50000
0
Response
$y = 1901x + 2169$
0
25
50
75
100
125
[analyte]

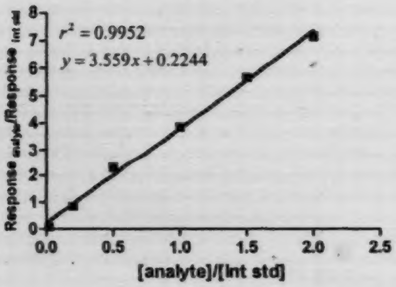
2) Internal standard calibration curves (for quantification of SPE extracts). Analyte concentrations, [analyte], are 1–100 μg/L. Internal standard concentration, [int std], is 50 μg/L. Line equation and r^2 values are displayed on plots.

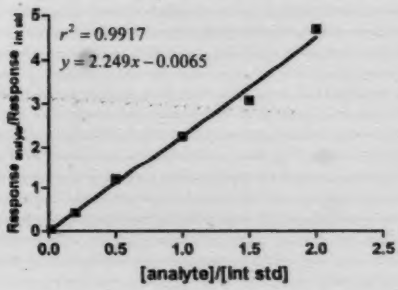

Sulfapyridine

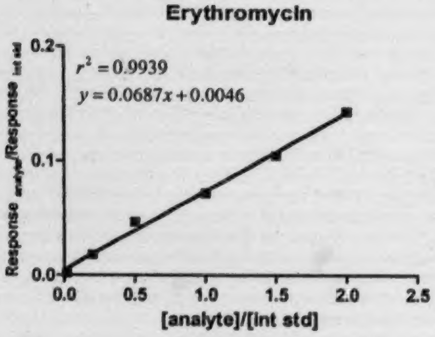

Sulfamethoxazole

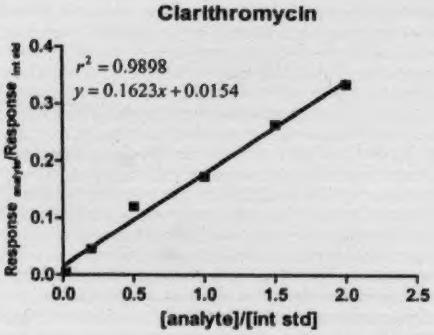

Sulfisoxazole

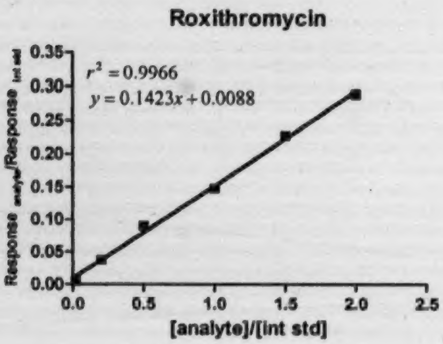

Sulfamethazine


Trimethoprim


Sulfadimethoxine


Erythromycin


Clarithromycin


Roxithromycin

3) Standard addition calibration curves (for quantification of SPME extracts). Spiked analyte concentration, [analyte], is in ng/mL. Analyte spike concentrations are 0–5 ng/mL. Line equation and r^2 values are displayed on plots.

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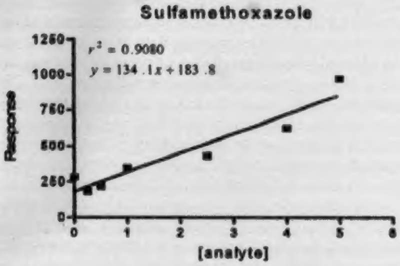
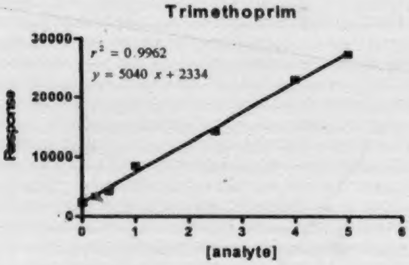
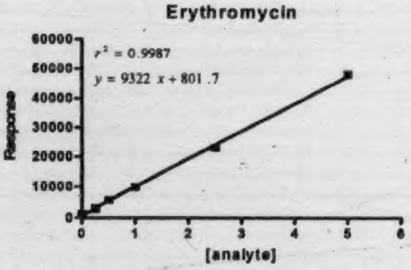
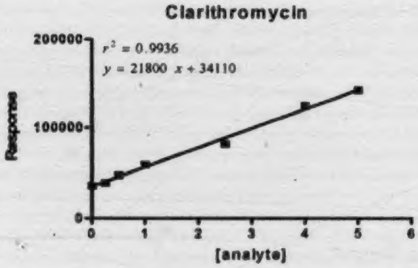
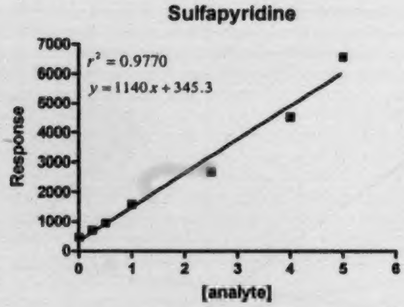
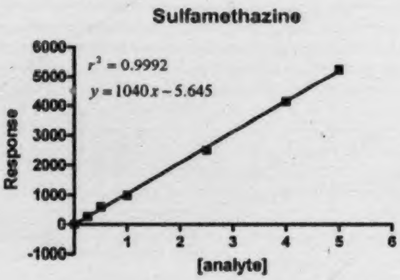

Sulfamethoxazole

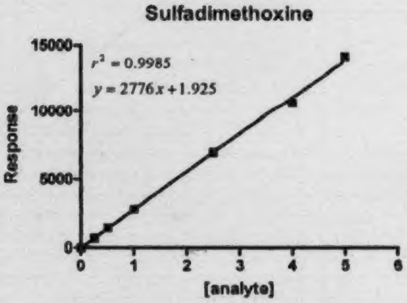

Trimethoprim

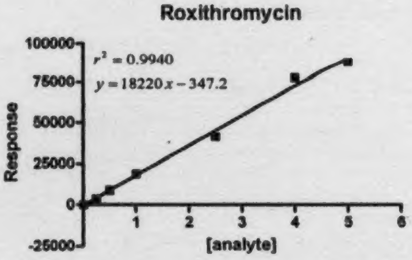

Erythromycin


Clarithromycin


Sulfapyridine


Sulfamethazine


Sulfadimethoxine


Roxithromycin

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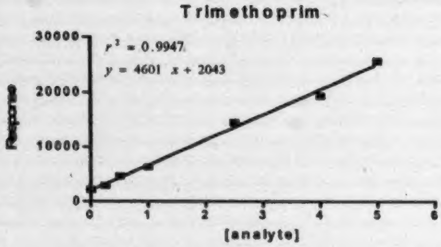
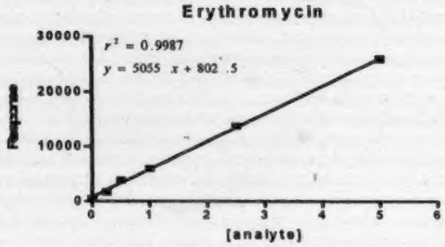
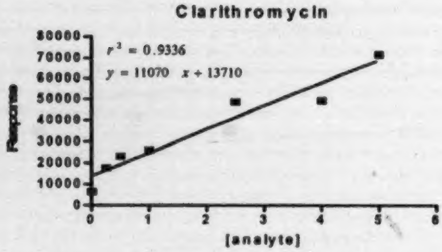
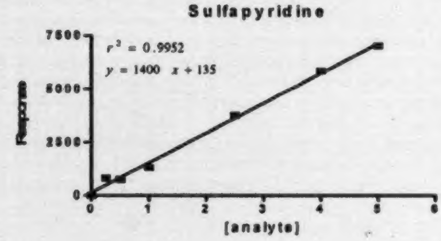
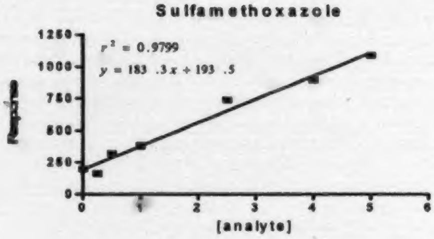
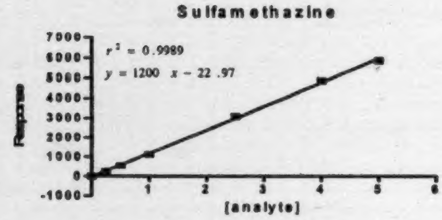

Trimethoprim

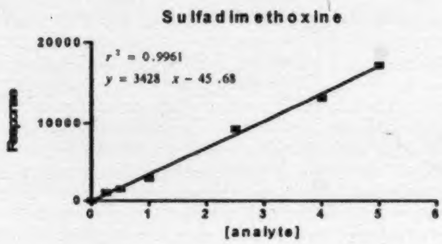

Erythromycin

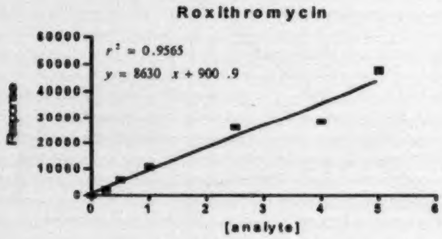

Clarithromycin


Sulfapyridine


Sulfamethoxazole


Sulfamethazine


Sulfadimethoxine


Roxithromycin

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